Introduction

MODERATOR: *W. Scott Helton, M.D., Division of General Surgery, University of Illinois, Chicago, Illinois*

PARTICIPANTS: *Robin S. McLeod, M.D., Justin Evans, M.D., University of Toronto, Toronto, Ontario, Canada; Robin Phillips, M.S., F.R.C.S., London, UK; Richard L. Nelson, M.D., F.A.C.S., University of Illinois College of Medicine at Chicago and University of Illinois School of Public Health, Chicago, Illinois; John F. Johanson, M.D., M.Sc., University of Illinois College of Medicine at Rockford and Rockford Gastroenterology Associates, Rockford, Illinois; Anthony J. Senagore, M.D., Cleveland Clinic Foundation, Cleveland, Ohio; James Fleshman, M.D., Washington University School of Medicine, St. Louis, Missouri*

This past spring, during Digestive Disease Week, The Society for Surgery of the Alimentary Tract (SSAT) hosted a consensus conference on benign anorectal disease in collaboration with the Society of American Gastrointestinal Endoscopic Surgeons (SAGES) and the American Society for Gastrointestinal Endoscopy (ASGE).

The consensus panel included six invited speakers: Robin S. McCleod, M.D., University of Toronto; Robin Phillips, M.D., St. Mark's Hospital, London; Richard L. Nelson, M.D., University of Illinois; John F. Johansen, M.D, Rockford, Illinois; Anthony J. Senagore, M.D., The Cleveland Clinic; and James Fleschman, M.D., Washington University; two discussants: Walter Koltun, M.D., Hershey, Pennsylvania; and Herand Abcarian, M.D., University of Illinois (Chicago); and members of the SSAT education and research committee: W. Scott Helton, M.D., Wiley Souba, M.D., Aron Fink, M.D., and Carlos Fernandez del-Castillo. During the first half of the conference, the invited speakers were asked to review the published literature and objectively evaluate evidence in favor of, or against, a given approach to management of chronic anal fissure. Specifically, the speakers were asked to address the following questions: (1) what is the optimal nonsurgical management for anal fissure; (2) what is the optimal surgical management for anal fissure; and (3) when is medical therapy inappropriate or ill advised for anal fissure? During the second half of the conference, the invited speakers were asked to present the medical evidence in favor of, or against, a given approach to the management of symptomatic internal hemorrhoids. Specifically, the speakers were asked to address the following questions: (1) what, if any, nonsurgical therapy is most efficacious and durable; (2) when is medical therapy inappropriate or ill advised; (3) what is the optimal timing and type of surgical therapy; and (4) what data exist on the safety, efficacy, and cost of using advanced technology to treat hemorrhoids? Finally, all speakers and panelists discussed what clinical trials should be conducted to answer the preceding questions? The final consensus statement was based on the evidence presented by the speakers and after a thorough discussion by the discussants, members of the audience, and the SSAT research committee.

1091-255X/02/$—see front matter
PII: S1091-255X(01)00077-4

Symptomatic Care and Nitroglycerin in the Management of Anal Fissure

Robin S. McLeod, M.D, Justin Evans, M.D.

Fissure in ano is a common condition seen by primary care physicians and surgeons. Although the pathogenesis of anal fissure remains incompletely understood, current hypotheses suggest that an anal fissure is an ischemic ulcer caused by the combination of spasm in the internal anal sphincter and relative ischemia in the posterior midline of the anal canal. Anatomic and postmortem angiographic studies have shown that blood vessels supplying the anal mucosa pass through the muscle of the internal anal sphincter. In the setting of a hypertonic sphincter, the blood flow to the anoderm is impaired, particularly in the posterior midline where there is a paucity of blood vessels.[1–3] Furthermore, an inverse correlation between anal tone and blood flow to the anoderm has been observed by Schouten et al.[4] Manometric studies have shown that fissures occur in patients with high resting anal pressures and ultraslow pressure wave activity in the internal anal sphincter. It is unclear whether spasm is a response to severe pain or if it has a pathogenic role in fissure formation.

The optimal treatment for chronic anal fissure has been to decrease the anal canal resting pressure by surgically dividing the internal sphincter to allow healing of the fissure. Recently there has been interest in pharmacologic agents that cause smooth muscle relaxation. These agents have the advantage of not causing permanent disruption of the normal sphincter function. Nitric oxide is the principal neurotransmitter mediating neurogenic relaxation of the internal anal sphincter. Glyceryl trinitrate (GTN) acts as a nitric oxide donor and is available as an ointment for topical administration. Topical GTN diffuses across the cutaneous barrier, causes a reduction in internal anal sphincter pressure, and improves anodermal blood flow. This is believed to be the mechanism by which GTN aids fissure healing. Studies have shown that GTN effectively reduces mean resting anal pressure.[5–7]

A total of nine randomized controlled trials have studied the efficacy of GTN in chronic anal fissure: five comparing GTN to placebo; one comparing GTN with botulinum toxin; and three comparing GTN with lateral internal sphincterotomy[5,6,8–14] (Table 1). In four out of five trials, GTN was significantly more effective than placebo in healing fissures.[5,6,8,9,11] The healing rates in the GTN group varied from 46% to 70%, and healing rates in the placebo group ranged from 8% to 51%. All studies used 0.2% GTN, except for the trial conducted by Carapeti et al.,[11] which tested escalating doses of GTN and found that 0.2% was as effective as 0.6%. It is not apparent why one study did not show a healing advantage for GTN over placebo, but it may have been due to the high rate of healing in the placebo group (51%).[8] In each of these trials, follow-up was short (1 month to 10 weeks). However, two trials have subsequently reported results of long-term follow-up, with symptomatic recurrence rates ranging from 27% to 62%.[5,15]

Three trials compared 0.2% to 0.5% GTN to lateral sphincterotomy[12,13,16] (Table 2). Sphincterotomy was clearly superior in two of these trials. In the third study there was pseudorandomization of patients. In all three studies there was an unblinded outcome assessment. In each of these trials healing rates with GTN were much lower than in the other trials, with 39% to 45% eventually having to undergo surgery. Follow-up ranged from 4 weeks to 6 months.

Differences in reported healing rates may relate to the difficulty in administering a standardized dose to all patients. Most published studies of topical GTN have used 0.2% to 0.3% preparations, although concentrations of up to 2% have been reported.[7] Because GTN is an unstable compound, some critics claim that not all patients are using active preparations. However, given the frequency of side effects, this is an unlikely explanation. Dosing instructions may include vague descriptions such as apply a "pea-sized" drop of ointment to the perianal skin or anal canal. Dosing frequencies vary from two to three times a day, and durations vary from 4 to 8 weeks.

From the Department of Surgery (R.S.M., J.E.), University of Toronto, Toronto, Ontario, Canada.
Address correspondence to: Robin S. McLeod, M.D., Mount Sinai Hospital, 600 University Ave., #449, Toronto, Ontario M5G 1X5, Canada.
e-mail: rmcleod@mtsinai.on.ca

Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(01)00078-6

Table 1. Randomized trials of glyceryl trinitrate (0.2%) vs. placebo for chronic anal fissures

Reference	No. of patients	Treatment alternative	Duration of therapy	Follow-up	Healing (%)		Headache (%)	
					GTN	Placebo	GTN	Placebo
Altomare et al.[8]	119	Placebo	4 wk	2 mo	49.2	51.7	33.8	7.8
Lund and Scholefield[6]	77	Placebo	8 wk	8 wk	68	8	57.8	17.9
Carapeti et al.[11]	68	1. Escalating dose of GTN (0.2% to 0.6%)	8 wk	10 wk	(0.2%) 65	32	72	27
		2. Placebo			(0.6%) 70			
Kennedy et al.[5]	43	Placebo	4 wk	4 wk	46	16	29	21
Bacher et al.[9]	35	Lignocaine gel	1 mo	1 mo	80	40	20	0

Healing rates may well be affected by alteration of any of these dosing variables.

The main concern with GTN is that a significant proportion of patients experience adverse effects. Headaches have been reported in 29% to 72%.[5,11] Lund et al.[17]observed headaches after application of 0.4% but not 0.2% GTN ointment to the anoderm of healthy volunteers. Most headaches were transient after application of GTN and were controlled with oral analgesics. Only 3% to 20% of patients discontinued treatment because of headaches.[5,6,12] Because healing rates are not improved with higher doses, and side effects become more common with escalating doses, the optimal concentration is probably 0.2%.

Tachyphylaxis is clinically relevant when cardiovascular disease is managed with nitrate therapy. Apart from one small study, there is no clinical evidence that an escalating dosage regimen is necessary when GTN is used to treat anal fissure.[18] Patients with a history of orthostatic hypotension, intolerance to nitrates, and ischemic heart disease have been excluded from most trials of GTN therapy and are probably best managed with an alternative therapy.

From these trials it seems that GTN is effective in healing one-third to two-thirds of patients with chronic anal fissure. With long-term follow-up, symptoms may recur in 38% to 60% of patients whose fissures were healed initially.[5,12] Therefore one strategy may be to treat patients initially with GTN, and thus avoid surgery in approximately 30%. However, Richard et al.[13] reported that at 6 weeks 86.8% of patients randomized to surgery were satisfied with their treatment, whereas only 46.3% of those randomized to GTN were satisfied. Nonrandomized studies have also shown increased side effects and reduced healing rates with GTN therapy.[19]

Alternative nitrates such as isosorbide dinitrate have been investigated, and healing rates of 80% to 90% have been observed in nonrandomized studies.[20,21] Mild transient headaches were reported in 17% to 100% of patients, although only one patient in the study by Lysy et al.[20] stopped treatment for this reason. Further investigation of these agents is required.

WHAT IS THE ROLE OF GLYCERYL TRINITRATE?

GTN is a useful first-line pharmacotherapy, although patients must be carefully instructed as to the appropriate use of this drug. It is labor intensive for both patients and physicians. Healing rates of 60% and recurrence rates of approximately 35% are an indication that for many patients GTN will, at best, be only a temporizing measure, and up to 50% will eventually require surgery. Other smooth muscle relaxants may be useful (for example, diltiazem) with

Table 2. Glyceryl trinitrate (0.2–0.5%) vs. surgical internal sphincterotomy for chronic anal fissure

Reference	No. of patients	Treatment alternative	Duration of therapy	Follow-up	Healing		Side effects	
					GTN	IS	GTN	IS
Richard et al.[13]	72	IS	6 wk	6 wk	29.5	89.5	84	28
				6 mo	27.2	92.1		
Evans et al.[16]	60	IS	8 wk	8 wk	60.6	97	NR	14
Oettle[12]	24	IS	4 wk	4 wk	83.3	100	NR	NR

IS = internal sphincterotomy; NR = not reported.

their improved side effect profiles, but more information is required before they can be advocated.

REFERENCES

1. Schouten WR, Briel JW, Auwerda JJ, et al. Ischaemic nature of anal fissure. Br J Surg 1996;83:63–65.
2. Lund JN, Binch C, McGrath J, et al. Topographical distribution of blood supply to the anal canal. Br J Surg 1999;86:496–498.
3. Klosterhalfen B, Vogel P, Rixen H, et al. Topography of the inferior rectal artery: A possible cause of chronic, primary anal fissure. Dis Colon Rectum 1989;32:43–52.
4. Schouten WR, Briel JW, Auwerda JJ. Relationship between anal pressure and anodermal blood flow. The vascular pathogenesis of anal fissures. Dis Colon Rectum 1994;37: 664–669.
5. Kennedy ML, Sowter S, Nguyen H, et al. Glyceryl trinitrate ointment for the treatment of chronic anal fissure: Results of a placebo-controlled trial and long-term follow-up [see comments]. Dis Colon Rectum 1999;42:1000–1006.
6. Lund JN, Scholefield JH. A randomised, prospective, double-blind, placebo-controlled trial of glyceryl trinitrate ointment in treatment of anal fissure [see comments] [published erratum appears in Lancet 1997;349:656]. Lancet 1997;349:11–14.
7. Loder PB, Kamm MA, Nicholls RJ, et al. 'Reversible chemical sphincterotomy' by local application of glyceryl trinitrate. Br J Surg 1994;81:1386–1389.
8. Altomare DF, Rinaldi M, Milito G, et al. Glyceryl trinitrate for chronic anal fissure—healing or headache? Results of a multicenter, randomized, placebo-controlled, double-blind trial. Dis Colon Rectum 2000;43:174–181.
9. Bacher H, Mischinger HJ, Werkgartner G, et al. Local nitroglycerin for treatment of anal fissures: An alternative to lateral sphincterotomy? Dis Colon Rectum 1997;40:840–845.
10. Brisinda G, Maria G, Bentivoglio AR, et al. A comparison of injections of botulinum toxin and topical nitroglycerin ointment for the treatment of chronic anal fissure. [see comments] [published erratum appears in N Engl J Med 1999;341:624] N Engl J Med 1999;341:65–69.
11. Carapeti EA, Kamm MA, McDonald PJ, et al. Randomised controlled trial shows that glyceryl trinitrate heals anal fissures, higher doses are not more effective, and there is a high recurrence rate. Gut 1999;44:727–730.
12. Oettle GJ. Glyceryl trinitrate vs. sphincterotomy for treatment of chronic fissure-in-ano: A randomized, controlled trial. Dis Colon Rectum 1997;40:1318–1320.
13. Richard CS, Gregoire R, Plewes EA, et al. Internal sphincterotomy is superior to topical nitroglycerin in the treatment of chronic anal fissure: Results of a randomized, controlled trial by the Canadian Colorectal Surgical Trials Group. Dis Colon Rectum 2000;43:1048–1058.
14. Tander B. Guven A, Demirbags S, et al. A prospective, randomized, double-blind, placebo-controlled trial of glyceryltrinitrate ointment in the treatment of children with anal fissure. J Pediatr Surg 1999;34:1810–1812.
15. Lund JN, Scholefield JH. Follow-up of patients with chronic anal fissure treated with topical glyceryl trinitrate [letter]. Lancet 1998;352:1681.
16. Evans J, Luck A, Hewett P Glyceryl trinitrate versus lateral sphincterotomy for anal fissure: A prospective, randomized trial. Dis Colon Rectum 2001;44:93–96.
17. Lund JN, Armitage NC, Scholefield JH. Use of glyceryl trinitrate ointment in the treatment of anal fissure. Br J Surg 1996;83:776–777.
18. Watson SJ, Kamm MA, Nicholls RJ, et al. Topical glyceryl trinitrate in the treatment of chronic anal fissure. Br J Surg 1996;83:771–775.
19. Hyman NH, Cataldo PA. Nitroglycerin ointment for anal fissures: Effective treatment or just a headache? [see comments] Dis Colon Rectum 1999;42:383–385.
20. Lysy J, Israelit-Yatzkan Y, Sestiere-Ittah M, et al. Treatment of chronic anal fissure with isosorbide dinitrate: Long-term results and dose determination. Dis Colon Rectum 1998;41: 1406–1410.
21. Schouten WR, Briel JW, Boerma MO, et al. Pathophysiological aspects and clinical outcome of intra-anal application of isosorbide dinitrate in patients with chronic anal fissure. Gut 1996;39:465–469.

Pharmacologic Treatment of Anal Fissure With Botoxin, Diltiazem, or Bethanechol

Robin Phillips, M.S., F.R.C.S.

Not all ulcers in the anal canal are idiopathic fissures. But for those patients who do have an idiopathic anal fissure, the etiology is considered to be a post-traumatic ischemic ulcer.[1] Some event, for example, passing a hard stool bolus, traumatizes the anal lining and causes pain. This is associated with a raised resting anal pressure associated with hypertonia of the internal anal sphincter, which in turn leads to poor vascular perfusion of the initially traumatic ulcer, leading to indolence and failure to heal. The predilection for idiopathic anal fissures to be situated in the posterior midline may simply reflect a vascular perfusion deficiency in this area, making it more susceptible to ischemia.[2] Classic treatment involves stool softeners and time, and with this approximately 30% of acute fissures will heal. However, some fissures become chronic.

WHAT IS A CHRONIC FISSURE?

There is no real agreement as to what constitutes a chronic anal fissure. Some have argued that it is a fissure that has persisted for at least 2 months. Others have used pathologic/anatomic signs as markers of chronicity, including exposure of fibers of the internal anal sphincter in the base and the local appearance of undermined edges. Lock and Thomson[3] examined the results of treatment of 188 consecutive new patients initially seen at St. Mark's Hospital in 1972 (when conservative treatment consisted of local anesthetic and an anal dilator). Where a fibrous anal polyp was present (31 patients), surgery was necessary in 84%; where a fibrous anal polyp was absent (157 patients), sphincterotomy was performed in only 48% ($P < 0.001$). Similarly, of the 73 patients with an anal skin tag, 73% needed an operation, whereas of the 115 patients without an anal skin tag, only 43% underwent sphincterotomy ($P < 0.001$). Thus a chronic anal fissure can also be defined as one having a fibroepithelial anal canal polyp or an associated skin tag. The reported results of nonoperative treatments will be markedly affected by which classification of chronicity is used.

SPHINCTEROTOMY AND WHY ALTERNATIVES ARE BEING DEVELOPED

The standard and very effective surgical treatments for anal fissure have for a long time been varieties of anal sphincterotomy, initially dorsal, then lateral, and then in some surgeons' practices, techniques of partial, bilateral sphincterotomy. The driving force for all of these changes was persistent reports of usually minor degrees of fecal incontinence. These were initially thought to be due to a gutter defect, when dorsal sphincterotomy was performed, and then, with unilateral full sphincterotomy, they were thought to be due to the fact that all of the internal sphincter was divided, which led to bilateral partial sphincterotomy. Current opinion supports full-thickness, partial, lateral sphincterotomy tailored to match the length of the fissure rather than a procedure designed to divide the whole of the internal sphincter.[4] Operative textbooks did, in the past, advocate division of the full length of the internal anal sphincter, and even those surgeons who tried not to do this may have been unaware of quite how short the internal anal sphincter usually is in women. Coupled with the high incidence of occult anal sphincter damage from vaginal delivery in women, lateral anal sphincterotomy can at times produce quite significant fecal incontinence. Khubchandani and Reed[5] discussed the results of 1355 patients who underwent sphincterotomy and found that 35% had flatus incontinence and 5% had accidental bowel movements. Some might consider this rate of incontinence to be rather high; perhaps a 5% risk of any sort of incontinence might be more generally accepted by most surgeons.

From St. Mark's Hospital (R.P.), London, UK.
Address correspondence to: Dr. Robin Phillips, St. Mark's Hospital, Watford Road, Harrow, Middlesex HA1 3UJ, UK. e-mail: gun@icrf.icnet.uk

1091-255X/02/$—see front matter
PII: S1091-255X(01)00079-8

As a consequence of this, it is now considered mandatory in some countries, including the United Kingdom, that patients be warned, at the time that consent is obtained for surgery, that there is a small but definite risk of flatus incontinence. In women with a complicated obstetric history, anal endosonography would seem a wise precaution before lateral anal sphincterotomy is performed.

DEVELOPMENT OF CHEMICAL SPHINCTEROTOMY

The neurotransmitter in the internal anal sphincter leading to relaxation is nitric oxide,[6] and nitric oxide donors cause reversible chemical sphincterotomy when applied to the anal margin of volunteers and patients with fissure.[7] Glyceryl trinitrate (0.2%) (GTN), applied to the anal margin,[8] is the most commonly employed agent. The rate of healing and side effects with GTN therapy for chronic anal fissure are discussed by Dr. Robin McLeod elsewhere in this issue.

ALTERNATIVE AGENTS

Botulinum Toxin

An alternative treatment has been the injection of botulinum toxin, which prevents the release of acetylcholine by presynaptic nerve terminals, in a dosage of 20 units of botulinum toxin A in two injections totaling 0.4 ml.[9] An earlier study using 15 units was shown to be less effective.[10] No sedation or local anesthesia has been necessary, and a 27-gauge needle has been used to inject the substance into the internal anal sphincter. Paralysis occurs within a few hours and lasts clinically for 3 or 4 months.

In a later randomized comparison between botulinum toxin (20 units) and nitroglycerin ointment (0.2% twice daily) in 50 patients, botulinum toxin was significantly more effective, healing 96% of the patients at 2 months vs. 60% healing with nitroglycerin ointment ($P = 0.005$).[11] But fissures tend to recur with time, and up to 10% of patients develop antibodies to botulinum toxin A,[12] particularly after repeated large injections, so the inherent repeatability and worry regarding using "one of the deadliest poisons known" will still make ointments attractive to doctors and patients alike.

Diltiazem and Bethanechol

The internal anal sphincter has a calcium-dependent mechanism and an extrinsic cholinergic innervation, both of which can be exploited to reduce resting anal pressure. Oral calcium channel blockers do work and reduce resting anal pressure, but the dosage required (60 mg) is approximately seven to eight times what is needed when the agent is applied topically (8 mg), which means topical treatment is much more likely to be free of any systemic side effects.[13]

The optimum dose of diltiazem gel is 2%, which has a maximal effect of 28% pressure reduction in the resting anal pressure, with the effect lasting from 3 to 5 hours. The optimal dose of bethanechol is 0.1%, producing a 24% pressure decrease. Ten of 15 fissures healed with 2% diltiazem gel and 9 of 15 with 0.1% bethanechol in open studies. No side effects were reported. The two treatments may be additive.[14]

QUESTIONS POSED

1. What is the optimal nonsurgical management for anal fissure? The answer is, botulinum; however, it is not conveniently available at the moment in a prepackaged Metred syringe, and there are questions with regard to repeatability. Of the creams, the side effect profile of diltiazem makes it the best choice.
2. What is the optimum surgical management for anal fissure? That depends on whether the anal fissure is the standard "high-pressure" anal fissure or one of the rarer "low-pressure" varieties. In the case of the former, the optimum surgical procedure is lateral anal sphincterotomy, the length of the sphincterotomy being tailored to the length of the fissure. However, for low-pressure anal fissures in women, an anal ultrasound examination should be performed, as some anterior low-pressure fissures can be a sign of an occult anterior sphincter injury, for which the treatment would be anterior sphincter repair.[15] If the anal ultrasound examination is normal, then some form of island flap operation should be attempted.
3. When is medical therapy inappropriate or ill advised for an anal fissure? In general, when an anal skin tag or a fibroepithelial anal canal polyp is present. In these circumstances, surgery is more likely to be effective in the long term. But patients will still need to be informed of the risk of flatus incontinence, and some so advised will opt for medical therapy. Otherwise, when there is a suggestion of an intersphincteric abscess opening into the base of the fissure, sphincterotomy should not be done.

Such an abscess can usually be palpated as a pea-sized swelling within the area of the fissure. This abscess should be treated by incision and drainage.

REFERENCES

1. Schouten WR, Briel JW, Auwerda JJ. Relationship between anal pressure and anodermal blood flow. The vascular pathogenesis of anal fissures. Dis Colon Rectum 1994;37:664–669.
2. Klosterhalfen B, Vogel P, Rixen H, et al. Topography of the inferior rectal artery: A possible cause of chronic, primary anal fissure. Dis Colon Rectum 1989;32:43–52.
3. Lock MR, Thomson JPS. Fissure-in-ano: The initial management and prognosis. Br J Surg 1977;64:355–358.
4. Richard C, Gregoire R, Plewes A, Silverman R, Burul C, Buie D, Reznick R, Ross T, Burnstein M, O'Connor B, Mukraj D, McLeod R. Internal sphincterotomy is superior to topical nitroglycerine in the treatment of chronic anal fissure: Results of a randomised controlled trial by the Canadian Colorectal Surgical Trials Group. Dis Colon Rectum 2000;43:1048–1058.
5. Khubchandani IT, Reed JF. Sequelae of internal sphincterotomy for chronic fissure in ano. Br J Surg 1989;76:431–434.
6. O'Kelly TJ, Brading AF, Mortensen NJ. Nerve-mediated relaxation of the human internal anal sphincter: The role of nitric oxide. Gut 1993;34:689–693.
7. Loder PB, Kamm MA, Nicholls RJ, Phillips RKS. Reversible chemical sphincterotomy by local application of glyceryl trinitrate. Br J Surg 1994;81:1386–1389.
8. Gorfine SR. Treatment of benign anal disease with topical nitroglycerin. Dis Colon Rectum 1995;38:453–457.
9. Maria G, Cassetta E, Gui D, Brisinda G, Bentivoglio AR, Albanase A. A comparison of botulinum toxin and saline for the treatment of chronic anal fissure. N Engl J Med 1998;338:217–220.
10. Gui D, Cassetta E, Anastasio G, Bentivoglio AR, Maria G, Albanase A. Botulinum toxin for chronic anal fissure. Lancet 1994;344:1127–1128.
11. Brisinda G, Maria G, Bentivoglio AR, Cassetta E, Gui D, Albanase A. A comparison of injections of botulinum toxin and topical nitroglycerin ointment for the treatment of chronic anal fissure. N Engl J Med 1999;341:65–69.
12. Hallett M. One man's poison—Clinical application of botulinum toxin. N Engl J Med 1999;341:118–120.
13. Carapeti EA, Kamm MA, Phillips RKS. Diltiazem lowers resting anal sphincter pressure—A potential low side-effect alternative to glyceryl trinitrate for fissures [abstr]. Gut 1998;42(Suppl):A97.
14. Carapeti EA, Kamm MA, Phillips RKS. Topical diltiazem and bethanechol decrease anal sphincter pressure and heal anal fissures without side effects. Dis Colon Rectum 2000;43:1359–1362.
15. Corby H, Donnelly VS, O'Herlihy C, O'Connell PR. Anal canal pressures are low in women with postpartum anal fissure. Br J Surg 1997;84:86–88.

A Review of Operative Procedures for Anal Fissure

Richard L. Nelson, M.D., F.A.C.S.

Operative techniques commonly used for anal fissure include anal stretch, open lateral sphincterotomy, closed lateral sphincterotomy, posterior midline sphincterotomy and, to a lesser extent, dermal flap coverage of the fissure. Reports of direct comparisons among these techniques are variable in their results and for the most part underpowered. Morbidity from these procedures, principally incontinence, was once thought to be extremely rare[1] but in recent reports has been found to be substantial,[2] further emphasizing the importance of making the correct operative choice. An analysis of the combined reports was therefore undertaken to determine whether a preferred technique for fissure surgery can be elucidated.

METHODS

Studies in which participants were randomized to one of two operative procedures are the primary focus of this review. Results of nonrandomized studies will also be reported, although they will be analyzed separately from the randomized studies. The subjects of this review are patients with chronic anal fissures. It is common practice among surgeons reporting this disease not to operate on acute fissures or fissures in children, or any atypical fissure (multiple, irregular, off the midline, or not associated with sphincter spasm). This series of restrictions has been specified in some but not all reports. The specific operative procedures included are anal stretch, open lateral internal sphincterotomy, closed lateral internal sphincterotomy, posterior midline sphincterotomy, and dermal flap coverage of the fissure. These procedures will be compared as follows: (1) anal stretch vs. sphincterotomy; (2) open vs. closed lateral sphincterotomy; and (3) posterior midline vs. lateral internal sphincterotomy. In the case of anal stretch vs. sphincterotomy, several different types of sphincterotomies were employed in various reports (i.e., open, closed, posterior). These were combined into a single sphincterotomy group in this first comparison. The two most significant end points are persistence of the fissure (which is used synonymously with persistence of anal pain, the measure of efficacy) and postoperative minor incontinence (the most commonly reported morbidity resulting from operations for anal fissure, also used synonymously with incontinence to flatus).

SEARCH STRATEGY FOR IDENTIFICATION OF STUDIES

The National Library of Medicine online PubMed search engine (www.nlm.nih.gov) was used to locate all published reports using the key words: "surgery, anal fissure." English language was not a restriction in the search. Additional search terms included "anal sphincterotomy," "anal fissure therapy," and "fissure therapy." The list of cited references in all included reports also was helpful in finding additional comparative studies, as was the Cochrane Library. All reports in which there was a direct comparison between at least two operative techniques were reviewed and when more than one report existed for any given pair, that report was included in the meta-analysis. If crude data were not presented in the report, the authors were contacted and crude data obtained. Crude data (number of patients experiencing each end point by procedure) were subjected to chi-square analysis from which the number of patients in each report, the confidence intervals, and the standard errors were obtained. MetaView software (www.cochrane.org) was used to evaluate the randomized studies only. In addition, both randomized and nonrandomized studies were employed in a meta-analysis in which the Mantel-Haenszel technique (EpiInfo6.04; www.cdc.gov) was used to compare results between the two types of studies. To assess homogeneity, MetaView was used in addition to the Mantel-Haenszel technique.[3]

RESULTS

Nineteen publications fulfilled the criteria of the study encompassing 3083 patients. The terminology

From the Department of Surgery (R.L.N.), University of Illinois College of Medicine at Chicago and the Epidemiology/Biometry Division (R.L.N.), University of Illinois School of Public Health, Chicago, Illinois.
Address correspondence to: Dr. Richard Nelson, Department of Surgery, 1740 West Taylor, Room 2204, M/C 957, Chicago, IL 60612. e-mail: altohorn@uic.edu

Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(01)00080-4

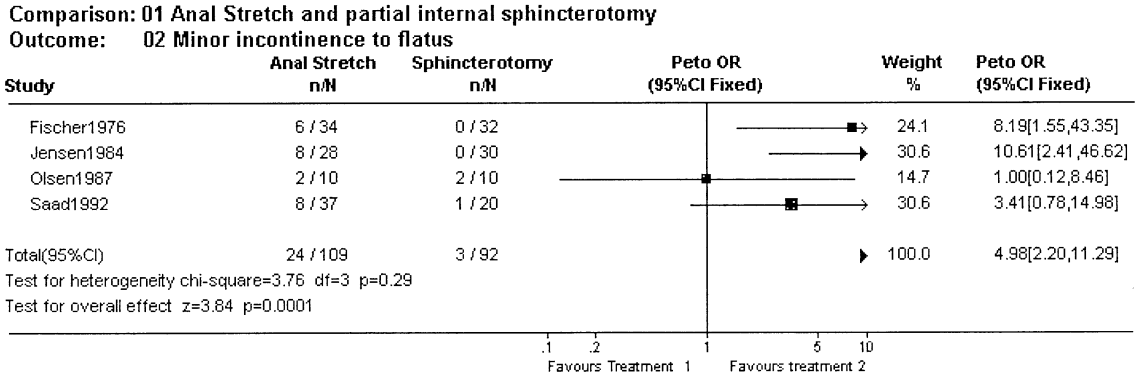


Fig. 1. MetaView box and whisker plot of a meta-analysis of randomized studies comparing anal stretch (treatment 1) to some form of internal sphincterotomy (treatment 2) and assessing fissure persistence or recurrence. This plot excludes two reports that had significant methodologic flaws.

used to describe end points varied from one report to another. Some were used synonymously in this review, such as fissure recurrence, fissure persistence, and need to reoperate on a patient with fissure. Also, incontinence was stratified in most reports from soiling, to incontinence to flatus, to incontinence to solid stool, whereas other reports just discussed minor incontinence, which is used synonymously with incontinence to flatus in this review. Duration of postoperative pain and time to healing were rarely reported. Ten of the reports prospectively randomized patients according to operative technique. However, one of these reports was reclassified as a retrospective report, since surgeons were described as routinely being allowed to change the procedure based on the operative findings.[4] The number of times this occurred in that report was not mentioned in the text.

A total of 11 reports compared anal stretch to internal sphincterotomy; five of these studies were retrospective and six were randomized. Incidence rates for persistence varied widely among reports from 5% to 30% in the anal stretch group and 3% to 29% in the internal sphincterotomy group (Table 1). Similarly, the rate of incontinence varied from 0% to 27% in the anal stretch group and from 0% to 20% in the sphincterotomy group (Table 2). There were seven reports comparing open and closed lateral internal sphincterotomies. Two of these were randomized studies and five were retrospective reviews (Tables 3 and 4). The rate of persistence in those reports comparing open and closed sphincterotomies varied from 0% to 9%, although incontinence in these reports varied from 1% to 30% (see Tables 3 and 4). There were four reports of retrospective reviews that provided data comparing posterior midline sphincterotomy to lateral sphincterotomy (Table 5) and one randomized report of this comparison.[10]

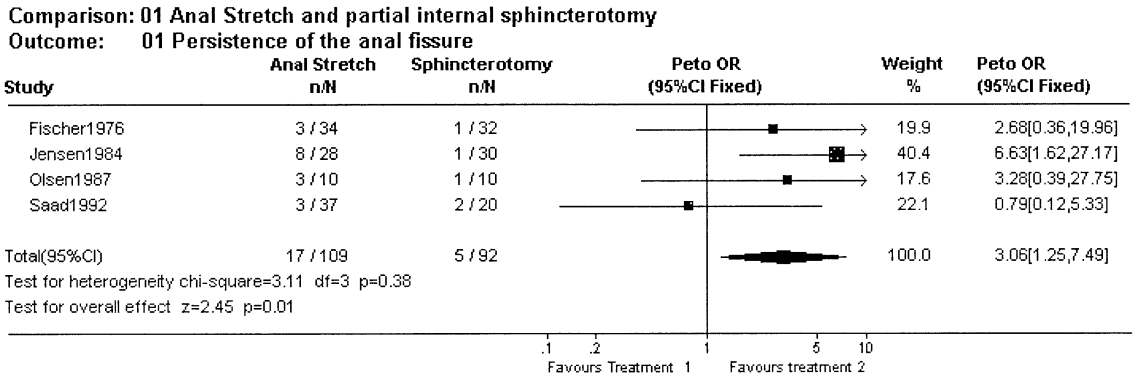


Fig. 2. MetaView box and whisker plot of a meta-analysis of randomized studies comparing anal stretch (treatment 1) to some form of internal sphincterotomy (treatment 2) and assessing minor incontinence after surgery. This plot excludes two reports that had significant methodologic flaws.

Table 1. Anal stretch vs. sphincterotomy

Reference	Anal stretch persistence risk	Sphincterotomy persistence risk	Anal stretch incontinence risk	Sphincterotomy incontinence risk
Randomized studies				
Jensen et al.[5] (n = 58)	0.27	0.04	0.27	0.00
Marby et al.[6] (n = 86)	0.10	0.29	0.00	0.00
Weaver et al.[7] (n = 98)	0.05	0.05	0.00	0.03
Olsen et al.[8] (n = 20)	0.30	0.10	0.20	0.20
Fischer et al.[9] (n = 66)	0.09	0.03	0.18	0.00
Saad and Omer[10] (n = 57)	0.08	0.05	0.24	0.05
Retrospective studies				
Giebel and Horch[11] (n = 238)	0.2	0.13	0.00	0.03
Collopy and Ryan[12] (n = 160)	0.30	0.15	0.24	0.15
Bekheit[13] (n = 65)	0.28	0.18	0.25	0.10
Hawley[4] (n = 74)	0.28	0.04	0.00	0.04
Hoffmann and Goligher[14] (n = 316)	0.16	0.05	0.13	0.06

Either midline or lateral sphincterotomy; some open, some closed.

METHODOLOGIC QUALITY OF INCLUDED STUDIES

Study Design

The principal quality grading in this review was separation of reports in which patients were randomized by operative technique vs. those reports that were retrospective reviews of experiences with two or more techniques. Separate analyses were carried out on the basis of study design, and the consistency of results was evaluated.

Randomization

Of the eight studies classified as randomized controlled trials, the technique of randomization was described in six: two by hospital registration number (even or odd),[7,10] three by pulling classification cards once it was determined that surgery was necessary,[6,15,16] and one by means of a random number table.[9]

Blinding

Only two studies report that physicians examining patients for recurrences or complications were blinded to patients' operative classifications.[6,9]

Completeness of follow-up is an additional quality measure. The dropout rate in which no follow-up occurred was determined. In addition, whenever possible, the likelihood that a significant number of randomized patients had not been followed long enough to reach the date at which end-point determination was to have occurred, according to the Methods section of that report, was also determined.

Six randomized studies compared the efficacy of anal stretch to some form of internal sphincterotomy, comprising 385 subjects.[5–10] Significant heterogeneity was detected in the analysis of efficacy with the MetaView software ($P = 0.032$). When two studies were deleted from the MetaView analysis (for

Table 2. Open vs. closed sphincterotomy

Reference	Open LIS persistence risk	Closed LIS persistence risk	Open LIS incontinence risk	Closed LIS incontinence risk
Randomized studies				
Kortbeek et al.[15] (n = 112)	0.06	0.03	0.14	0.21
Boulos and Araujo[16] (n = 28)	0.00	0.00	0.07	0.09
Retrospective studies				
Pernikoff et al.[17] (n = 312)	0.02	0.01	0.05	0.05
Garcia-Aguilar et al.[2] (n = 549)	0.05	0.64	0.30	0.24
Ektov et al.[18] (n = 154)	0.01	0.09	0.01	0.06
Lewis et al.[19] (n = 350)	0.08	0.05	0.06	0.07

CS = closed sphincterotomy; LIS = lateral internal anal sphincterotomy; OS = open sphincterotomy.
For studies with zero numerators in some cells, the relative risks (RR), standard errors (SE), and 95% confidence intervals (CI) listed are estimates of the lower limit (RR, CI) and upper limit (SE).

Table 3. Posterior midline vs. lateral internal sphincterotomy: Retrospective studies

Reference	PMS persistence risk	LIS persistence risk	PMS incontinence risk	LIS incontinence risk
Hawley[4] (n = 56)	0.06	0.00	0.06	0.00
Bekheit[13] (n = 40)	0.25	0.10	0.00	0.20
Abcarian[1] (n = 300)	0.02	0.02	0.05	0.00
Hoffman and Goligher[1] (n = 226)	0.07	0.03	0.19	0.06

LIS = lateral internal anal sphincterotomy; PMS = posterior midline sphincterotomy.
For studies with zero numerators in some cells, the relative risks (RR), standard errors (SE), and 95% confidence intervals (CI) listed are estimates of the lower limit (RR, CI) and upper limit (SE).

reasons discussed below[6,7]), the heterogeneity disappeared ($P = 0.38$), and the overall effect for efficacy became significant, favoring sphincterotomy (odds ratio = 3.08; 95% confidence interval [CI] = 1.26 to 7.54). All of these reports also looked at minor or flatus incontinence as a complication of each procedure. The Peto odds ratio was 4.22 and the 95% CI was 1.89 to 9.41) in favor of sphincterotomy. No heterogeneity was detected in the analysis of minor incontinence ($P = 0.1$) with or without the abovementioned two studies.

Two randomized studies compared open partial lateral internal sphincterotomy to closed or subcutaneous partial lateral internal sphincterotomy[15,16] in a total of 140 subjects. The same two end points were assessed: persistence of the fissure and partial or flatus incontinence. The Peto odds ratio for persistence of fissure was 1.63 and the 95% CI was 0.27 to 9.74 (favoring open sphincterotomy), and for flatus incontinence the odds ratio was 0.77 and the CI was 0.25 to 2.31 (favoring closed sphincterotomy).

One more report randomized patients to either sphincterotomy or dermal flap coverage of the anal fissure.[20] Three strata of outcome were reported from dissatisfied to satisfied to excellent. The outcome was generally better in the flap group (although not significantly so ($P = 0.23$ by chi-square analysis). However, the only recurrences developed in the flap group, in 3 of 20 patients. Incontinence was not seen in either group.

The nonrandomized studies included many more patients, but except for selection bias the ascertainment of end points was almost always retrospective. Nevertheless, the combined overall results of those reports (see Table 4) are quite similar to the findings in the randomized studies: a slight favoring of sphincterotomy over anal stretch and very little difference in results of open vs. closed sphincterotomy. Posterior internal sphincterotomy is more likely to result in persistence (not statistically significant) and incontinence to flatus than lateral internal sphincterotomy (see Table 4).

Homogeneity was found not to be present in three instances (see Table 5): the first in randomized trials is described earlier in this report; the second instance is found in retrospective studies of incontinence risk after open vs. closed sphincterotomy (see Table 2); and the third instance is found in the retrospective comparison of posterior and lateral sphincterotomy (see Table 3). The source of this heteroge-

Table 4. Surgery for fissure in ano: Summary statistics

Study design	RR persistence	95% CI	RR incontinence	95% CI
Anal stretch vs. sphincterotomy	2.15*	1.52–3.04	1.60*	1.01–2.54
Retrospetive studies	5 studies		5 studies	
Open vs. closed LIS	0.94	0.55–1.58	1.16	0.90–1.51
Retrospective studies	4 studies		4 studies	
PMS vs. LIS	2.12	0.93–4.73	2.94*	1.38–5.54
Retrospective studies	4 studies		4 studies	

LIS = lateral internal anal sphincterotomy; PMS = posterior midline sphincterotomy.
Sphincterotomies in various reports were either midline or lateral; some were open, some were closed. For studies with zero numerators in some cells, the relative risks (RR), standard errors (SE), and 95% confidence intervals (CI) listed are estimates of the lower limit (RR, CI) and upper limit (SE). Meta-analysis of comparative literature encompassed paired studies of four surgical techniques; retrospective studies measured two end points: persistence of the fissure and flatus incontinence.
*$P < 0.05$.

Table 5. Tests of homogeneity

Study design	Persistence	Incontinence
Anal stretch vs. sphincterotomy	$P = 0.6$	$P = 0.95$
Retrospective studies	5 studies	5 studies
Open vs. closed LIS	0.82	$P = 0.01$
Retrospective studies	4 studies	4 studies
PMS vs. LIS	0.8	$P < 0.01$
Retrospective studies	4 studies	4 studies

LIS = lateral internal anal sphincterotomy; PMS = posterior midline sphincterotomy.
Sphincterotomies in various reports were either midline or lateral; some were open, some were closed. For studies with 0 numerators in some cells, the values listed are estimates of the lower limit and upper limit (SE). Homogeneity is rejected when $P < 0.05$.

neity is readily apparent when individual studies are examined. One report describes a risk of minor incontinence (30% and 24%) after either open or closed sphincterotomy that greatly exceeds risk in other reports. These data were obtained from a mailed questionnaire in a large population.[2] It cannot be determined whether in this report the high rate was due to the method of ascertainment or the operative technique of sphincterotomy. The categorization of self-reporting of functional symptoms after an operation as a surgical complication (as was done in the study by Garcia-Aguilar et al.[2]) is a technique that requires validation.

Saad and Omer[10] assessed three procedures in a randomized trial (two posterior and one lateral sphincterotomy) involving 41 subjects. This was the only study to randomize these two procedures, and the results demonstrated little difference between these two procedures with regard to both efficacy (no recurrence with posterior and one with lateral sphincterotomy) and incontinence (two with incontinence after posterior and one after lateral sphincterotomy). The results of the nonrandomized studies of this comparison demonstrate a higher risk of incontinence to flatus with posterior sphincterotomy and can be seen in Table 3.

DISCUSSION

Among the randomized studies, heterogeneity is detected. The study by Marby et al.[6] is responsible for this, since removal of it from the meta-analysis eliminates the heterogeneity. Its results related to persistence or recurrence are clearly different from other reports. Scrutiny of this report reveals several problems. First is the dropout rate of 24%—that is, randomized individuals not appearing for follow-up. In addition, the mean follow-up time compared to the range of follow-up times implies a significant skewing to the right of this set, with clustering of individuals below the mean, many of whom were followed up for too little time to accurately determine the end points. Only 31 of 156 patients were evaluated at 12 months. In addition, there is another variable inserted between the two groups besides operation and that is anesthesia—that is, the stretch group had general anesthesia and the sphincterotomy group had only local anesthesia. Further difficulties with this study relate to the indications for operation. The presenting symptom of fissure is, except in very unusual circumstances, anal pain on defecation, and all treatments are directed toward pain relief. Yet 27% of patients in this report did not have pain as a presenting symptom. Furthermore, for the sphincterotomy group to have worse results than the stretch group implies that the sphincterotomies were not adequately performed in this study when compared to other reports. This is further implied by the postoperative manometric findings reported by Marby et al.[6] The patients in whom healing of the fissure did not occur (14 of 17 cases) had significantly elevated sphincter pressure, denoting incomplete sphincterotomy. These same investigators conducted a subsequent randomized trial,[7] the results of which differed markedly from those of the first trial. The anesthesia was now the same in both groups and perhaps the sphincterotomy was more complete, although this was not directly evaluated as it had been in several other reports.[5,6,8,9] However, significant problems with dropouts and follow-up persist in this second report. The dropout rate was 14% and again, when the mean and range of follow-up are examined, a large percentage of the patients could not have been seen at 12 months (because the paper was written too soon), although this end point was specified in the Methods section. The dropout rate for all other randomized studies varied from 0% to 1%. Follow-up was also to the specified end-point date for all patients in other randomized studies. For all these reasons it is reasonable to consider analysis of the studies in the absence of the reports by Marby et al.[6] and Weaver et al.,[7] when evaluating anal stretch and sphincterotomy, both in terms of their effectiveness in curing anal fissure and the risk of minor fecal incontinence after surgery.

Anal stretch has a higher risk of persistence of fissure compared to internal sphincterotomy and also carries a significantly higher risk of minor incontinence compared to sphincterotomy (Figs. 1 and 2). The combined results of open vs. closed partial lateral internal sphincterotomy show little difference between the two procedures, both in persistence of fissure and risk of incontinence. Therefore the use of

anal stretch in the treatment of chronic anal fissure in adults should probably be abandoned. For those patients requiring surgery for anal fissure, open and closed partial lateral internal sphincterotomies appear to be equally efficacious. It is less clear whether posterior sphincterotomy should be performed as the primary treatment for anal fissure.

REFERENCES

1. Abcarian H. Surgical correction of chronic anal fissure: Results of lateral internal sphincterotomy vs. fissurectomy—midline sphincterotomy. Dis Colon Rectum 1980;23:31–36.
2. Garcia-Aguilar J, Belmonte C, Wong WD, Lowry AC, Madoff RD. Open vs. closed sphincterotomy for chronic anal fissure: Long term results. Dis Colon Rectum 1996;39:440–443.
3. Petitti DB. Meta-analysis, Decision Analysis and Cost Effective Analysis. Oxford: Oxford Univeristy Press, 1994.
4. Hawley P. The treatment of chronic fissure in ano: A trial of methods. Br J Surg 1969;56:915–917.
5. Jensen SL, Lund F, Nielsen OV, Tange G. Lateral subcutaneous sphincterotomy versus anal dilatation in the treatment of fissure in ano in outpatients: A prospective randomized study. BMJ 1984;289:528–530.
6. Marby M, Alexander-Williams J, Buchman P, et al. A randomized controlled trial to compare anal dilatation with lateral subcutaneous sphincterotomy for anal fissure. Dis Colon Rectum 1979;22:308–311.
7. Weaver RM, Ambrose NS, Alexander-Williams J, Keighley MR. Manual dilatation of the anus vs. lateral subcutaneous sphincterotomy in the treatment of chronic fissure in ano. Results of a prospective, randomized clinical trial. Dis Colon Rectum 1987;30:420–423.
8. Olsen J, Mortensen PE, Krogh-Petersen I, Christiansen J. Anal sphincter function after treatment of fissure-in-ano by lateral subcutaneous sphincterotomy versus anal dilatation. A randomized study. Int J Colorectal Dis 1987;2:155–157.
9. Fischer M, Thermann M, Trobisch M, Sturm R, Hamelmann H. Die behandlung der primar-chronischen analfissur durch dehnung des analkanales oder sphincterotomie. Langenbecks Arch Chir 1976;343:35–44.
10. Saad AM, Omer A. Surgical treatment of chronic fissure-in-ano: A prospective randomized study. East Afr Med J 1992;69:613–615.
11. Giebel GD, Horch R. Treatment of anal fissure: A comparison of three different forms of therapy. Nippon Geka Hokan 1989;58:126–133.
12. Collopy B, Ryan P. Comparison of lateral subcutaneous sphincterotomy with anal dilatation in the treatment of fissure in ano. Med J Aust 1979;2:461–462.
13. Bekheit F. Anal fissure: Comparative study of various lines of treatment in 125 cases. J Egypt Med Assoc 1974;57:365–372.
14. Hoffmann DC, Goligher JC. Lateral subcutaneous internal sphincterotomy in treatment of anal fissure. Br Med J 1970;3:673–675.
15. Kortbeek JB, Langevin JM, Khoo RE, Heine JA. Chronic fissure-in-ano: A randomized study comparing open and subcutaneous lateral internal sphincterotomy. Dis Colon Rectum 1992;35:835–837.
16. Boulos PB, Araujo JG. Adequate internal sphincterotomy for chronic anal fissure: Subcutaneous or open technique? Br J Surg 1984;71:360–362.
17. Pernikoff BJ, Eisenstat TE, Rubin RJ, Oliver GC, Salvati EP. Reappraisal of partial lateral internal sphincterotomy. Dis Colon Rectum 1994;37:1291–1295.
18. Ektov VN, Nalivkin AI, Kriachkov AA, Lakatosh KO. Selection of the most effective method of lateral subcutaneous sphincterotomy in the treatment of fissure in ano (in Russian). Vestn Khir 1986;136:37–41.
19. Lewis TH, Corman ML, Prager ED, Robertson WG. Long-term results of open and closed sphincterotomy for anal fissure. Dis Colon Rectum 1988;31:368–371.
20. Leong AF, Seow-Choen F. Lateral sphincterotomy compared with anal advancement flap for chronic anal fissure. Dis Colon Rectum 1995;38:69–71.

Nonsurgical Treatment of Hemorrhoids

John F. Johanson, M.D., M.Sc.

Hemorrhoids begin as localized cushions of specialized submucosal vascular tissue located in the anal canal adjacent to the junction of the squamous and columnar epithelium. These cushions are present at birth and represent a normal anatomic feature of the anal canal. The existence of hemorrhoidal cushions alone, therefore, does not constitute disease. Hemorrhoidal disease requires the presence of pathologic changes leading to bleeding, prolapse, or thrombosis.

Rectal bleeding is the most common manifestation of hemorrhoidal disease.[1] The bleeding typically appears as a bright red spot or streak on the toilet tissue or stool but may also be seen dripping into the toilet water. If prolapse is the predominant symptom, a mass may be sensed protruding through the anus with defecation. Early in its course, prolapse reduces spontaneously. In time, chronic prolapse results in persistent mucoid discharge, which may cause perianal irritation and/or pruritis. Pain is not typically a symptom of internal hemorrhoid disease unless the hemorrhoids are acutely prolapsed, which requires immediate surgical intervention. Pain may also be associated with thrombosed external hemorrhoids.

Hemorrhoids are one of the most common gastrointestinal disorders affecting nearly 5% of the United States population. More than 10 million persons annually admit to symptoms of hemorrhoidal disease. However, only one third of them are ever seen by a physician for evaluation or treatment of this condition.[2] Therapy can be broadly categorized into oral therapy such as fiber, topical treatments, nonsurgical destructive techniques, and surgical intervention. The decision to treat is based on the frequency and severity of symptoms. Those without symptoms of bleeding or prolapse or with infrequent symptoms often do not require any therapy, particularly since the definition of hemorrhoidal disease is based on the presence of symptoms. The specific treatment also depends to a great extent on the nature of the patient's symptoms. There is no disagreement, for example, that acutely prolapsed hemorrhoids require surgical intervention. However, the overwhelming majority of patients with symptomatic hemorrhoids do not present in this manner. It is the population of patients with intermittent but bothersome symptoms that requires elucidation of the optimal therapy.

CONSERVATIVE TREATMENT

In routine clinical practice, the initial choice for treatment of hemorrhoidal disease is generally conservative, including both oral and topical therapies. This approach is recommended for persons with minor symptoms that do not interrupt normal daily activities. The goal of medical treatment is to relieve symptoms as quickly as possible. A secondary goal is to maintain remission of symptoms. Treatment of the underlying pathophysiologic cause of symptoms is not possible nor is it intended with these modalities. Options for oral therapy include fiber supplements and oral diosmin (Daflon). Although fiber is widely recommended in this situation, the data supporting this option are inconsistent. One controlled trial has been published comparing fiber (psyllium seed) with placebo in 52 nonselected patients with symptomatic hemorrhoidal disease.[3] In this trial, fiber supplements were given for 6 weeks and resulted in significantly reduced bleeding when compared with placebo; 92% vs. 56%, respectively. This provided an absolute risk reduction in bleeding associated with treatment of 36% and a number needed to treat (NNT) of 2.8. This means that nearly three patients will need to be treated to achieve one symptomatic response. Pain with defecation was likewise reduced with fiber supplements demonstrating response rates of 96% vs. 68%. This corresponded to a risk reduction of 28% with an NNT of 3.6. A less impressive reduction in pruritis and prolapse was noted, which failed to reach statistical significance. By contrast, another clinical trial comparing a different form of fiber supplementation demonstrated no initial difference between fiber and placebo with regard to bleeding during the first 15 days of treatment.[4] In the subsequent 3 weeks, a small difference was observed between treatment and control groups,

From the University of Illinois College of Medicine at Rockford (J.F.J.) and Rockford Gastroenterology Associates (J.F.J.), Rockford, Illinois. Address correspondence to: Dr. John F. Johanson, Rockford Gastroenterology Associates, 401 Roxbury Rd., Rockford, IL 61107. e-mail: johnfj@uic.edu

Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(01)00081-6

but the magnitude of treatment effect was less than what was observed in the study described above. Furthermore, there was no difference in prolapse among the two groups. Finally, two additional clinical trials examining other forms of fiber have shown no clear proof of efficacy.[5,6]

The data supporting the efficacy of oral Daflon is even less convincing. Two controlled trials of Daflon observed improvement in the treatment group when compared to placebo.[7,8] These trials included 120 and 100 patients, respectively. Improvement was noted in subjective symptom scores for pain and anal discharge. In the first study, prolapse was likewise reduced. However, a third trial of 100 patients conducted in Thailand showed no added improvement with Daflon vs. placebo when added to a conservative regimen of fiber supplementation.[9] In the latter study there was no significant difference in the number of patients with subjective improvement of symptoms. Overall response rates were 94% and 98% (NS) for fiber alone compared to fiber and Daflon, respectively. These results, in fact, seem to support the efficacy of fiber therapy and suggest that Daflon adds little. Unlike the other clinical trials of fiber supplements described earlier, however, this trial did not have a true placebo control group.

The evidence supporting the beneficial effect of fiber supplements in eliminating the acute symptoms of hemorrhoidal disease is not conclusive because of the small numbers of patients examined and the seemingly conflicting results. Nevertheless, fiber is safe and inexpensive. Fiber therapy in all likelihood, therefore, will remain an integral part of the initial treatment of hemorrhoidal disease, even in the absence of undeniable proof.

Topical therapy is the other first-line therapeutic alternative to treating the acute symptoms of hemorrhoidal disease. A large number of options are available, although the evidence supporting their efficacy is weak. Topical therapies reduce symptoms by exerting a local anesthetic effect, which eliminates the burning and itching associated with hemorrhoid prolapse. They have less of an effect on bleeding, although they are frequently used for this indication.

A number of topical preparations are available and are listed in Table 1. There have been no clinical trials supporting the efficacy of any of these products. The only compound in which a randomized clinical trial was performed was 5-ASA suppositories.[10] In this case, a double-blind placebo-controlled trial demonstrated significant reductions in hemorrhoidal symptoms among patients treated with 5-ASA suppositories compared with placebo. Analogous to fiber supplements, topical therapy has few side effects and is generally safe. Long-term use of topical steroids, however, has the potential to cause chronic perianal dermatitis.

NONSURGICAL TREATMENT OPTIONS

Bleeding or other symptoms that persist despite conservative management require more aggressive therapy aimed at eliminating the underlying pathophysiologic abnormality. It is in this group that the most controversy arises when attempts are made to identify the best type of treatment, that is, nonsurgical options or surgical hemorrhoidectomy. The remainder of this discussion will focus on the question of which of these treatment modalities should be the optimal choice for chronically symptomatic hemorrhoid disease.

The underlying goal of nonsurgical therapy is fixation of the hemorrhoidal cushion. The most common methods currently being employed are injection sclerotherapy, rubber band ligation (RBL), and infrared photocoagulation (IRC).[11] Injection sclerotherapy for this purpose has been in use the longest; it was first employed more than 100 years ago in Europe. In this method, a small amount of sclerosant is injected into the submucosa above the hemorrhoid cushion, leading to fibrosis and ultimate fixation of the hemorrhoid cushion. RBL is probably the most commonly used nonsurgical treatment for hemorrhoidal disease. A special applicator is used to place one or more rubber bands at the base of each hemorrhoid cushion, strangulating a small amount of mucosa with the cushion. This technique has recently been adapted to allow placement of rubber bands endoscopically. The underlying mode of action, however, remains the same. The most recent advance in nonsurgical treatment of hemorrhoidal disease is the use of "heat" delivered in a variety of forms including infrared light or electrocautery. IRC is the best known and most widely studied technique, which causes tissue destruction by a rapid increase in heat delivered by an infrared light source.

Despite the widespread application of these methods, there have been no placebo-controlled trials to establish their efficacy. Most of the studies assessing the benefits of these techniques have been descriptive studies or case series, which have detailed the effects of patients undergoing the specific treatment being described. None of these methods, however, has been compared with conservative therapy or placebo in randomized, controlled clinical trials. Without such comparisons, it is impossible to know whether the observed response was truly the result of treatment or simply related to the natural history of hemorrhoidal disease.

Notwithstanding the absence of placebo-controlled trials, there have been a number of randomized trials comparing these various modalities with each other.[12–19] Despite the abundance of nonsurgical options for the

Table 1. Topical therapies for treatment of hemorrhoidal disease

Agent	Mechanism of action	Dosage	Benefits	Side effects	Comments
Pramoxine HCl 1% (Anusol ointment)	Rapidly acting local anesthetic	Apply topically up to 5 times daily	Local symptom relief; no cross-reactivity with other local anesthetics such as procaine or dibucaine	Local allergic reaction	Indicated for temporary relief of soreness, burning, or itching; forms a temporary protective coating over the inflamed tissues
Hydrocortisone acetate 1% (Anusol HC suppositories)	Topical anti-inflammatory agent	Use up to b.i.d.	Useful in treatment of internal hemorrhoids	Local allergic reaction	Unlike topical creams, suppositories provide therapy further up into the anal canal for treatment of bleeding or prolapsing internal hemorrhoids
Hydrocortisone acetate 1% (Cortaid cream)	Topical anti-inflammatory agent	Use topically up to q.i.d.	Local symptom relief; steroid provides anti-inflammatory properties	Local allergic reaction	
Phenylephrine HCl 0.25% (Preparation H, cream, gel, or suppositories)	Local vasoconstriction leading to temporary relief of burning and itching	Use topically or per rectum up to q.i.d.	Works by different mechanism than other topical creams; may be effective if other local anesthetics are not effective	Local allergic reaction	
5-amino salicyclic acid suppositories	Local anti-inflammatory effect		Reduces pain and bleeding of hemorrhoids		Fewer side effects than topical steroid therapy

treatment of hemorrhoidal disease, none has consistently been shown to be better than the others. The absence of a clear advantage may indicate that they are equally effective. A more likely explanation, however, is that no clear choice has emerged because of the lack of statistical power among the various small clinical trials to demonstrate a significant difference among treatment options.

To address this problem, we performed a meta-analysis comparing the three most common nonsurgical techniques: IRC, RBL, and injection sclerotherapy.[20] Seven clinical trials were found comparing the three techniques, but two were eliminated because of serious problems with their methodology. Thus five randomized, controlled clinical trials were considered in the meta-analysis: two comparing RBL with IRC, two comparing sclerotherapy with RBL, and one study that compared all three treatments. When the studies were combined, pooled analyses revealed similar efficacy between RBL and IRC, whereas sclerotherapy was not as efficacious. Although initial efficacy between RBL and IRC was similar, significantly fewer patients undergoing RBL required additional treatments for symptom recurrence. This therapeutic advantage, however, occurs in the presence of a fivefold greater incidence of treatment-related pain compared with IRC.

The results of the meta-analysis have subsequently been confirmed by a randomized controlled

trial of 133 consecutive patients.[21] In this trial 97% of patients treated with RBL and 92% of patients treated with IRC were symptom free or had improved to a satisfactory degree by the end of treatment (not significant [NS]). Similar to what was observed in our meta-analysis, RBL was associated with significantly more pain. In contrast to the meta-analysis, no differences in the long-term effects of IRC and RBL were observed. Given comparable efficacy and a significantly higher risk of pain, IRC was recommended as the preferred choice for nonsurgical therapy of hemorrhoidal disease.

A second meta-analysis was performed in 1995 by MacRae and McLeod[22] that used many of the same clinical trials. Not surprisingly, this meta-analysis showed nearly identical results, although the authors of this study came to a different conclusion. In this study RBL and IRC again demonstrated similar efficacy and were more efficacious than sclerotherapy. Patients treated with IRC were more likely to require repeat treatment, whereas RBL was significantly more painful. RBL, however, was recommended as the optimal therapy presumably because of its increased efficacy (less need for retreatment), even though it was associated with significantly more patient discomfort. It is undeniable that the difference in efficacy between RBL and IRC is limited, and therefore the choice of the optimal therapy should be individualized when possible. That is, patients should be asked whether they are willing to endure more discomfort with the initial therapy or opt for the treatment that causes less pain but may require repeat therapy in the future. I suspect most patients will choose the latter.

COMPARISON OF SURGICAL HEMORRHOIDECTOMY WITH NONSURGICAL TREATMENT

Unfortunately, there is little evidence from clinical trials to guide us in answering this question as well. The majority of the clinical trials comparing surgical with nonsurgical treatment options were performed in the early 1980s. The applicability of these trials to current practice is unclear, given the improvements in surgical techniques and the emergence of thermal fixation methods such as infrared coagulation, which were not widely available at the time these direct comparative trials were being performed.

The best evidence available examining the efficacy of surgical vs. nonoperative treatment comes from the meta-analysis performed by MacRae et al.[22,23] as described earlier in this report. Overall, 18 randomized controlled trials were examined and included in the meta-analysis. Of these, three directly compared hemorrhoidectomy with RBL, the only nonsurgical technique to be tested. Of these individual trials, one demonstrated hemorrhoidectomy to be more effective,[24] whereas the other two were inconclusive.[25,26] When the results of the individual trials were pooled, hemorrhoidectomy was found to be significantly more effective than RBL but was also associated with a significantly greater risk of complications and pain. Although hemorrhoidectomy was clearly more efficacious, the authors believed that RBL should be the initial choice for treatment of hemorrhoidal disease. Surgery, in their opinion, should be reserved for those patients in whom nonoperative procedures are unsuccessful.

SUMMARY

Although symptomatic hemorrhoidal disease affects nearly 5% of the United States population, it is disappointing that only a relatively few well-designed, randomized, placebo-controlled clinical trials have been performed. Despite the absence of unequivocal evidence, I believe a number of conclusions can be reliably drawn. First, the preponderance of evidence supports the efficacy of fiber supplements in eliminating the acute symptoms of hemorrhoidal disease. Second, despite their widespread use, there is little to support the efficacy of topical therapy. Third, IRC and RBL demonstrate comparable efficacy, although RBL is probably slightly better for treatment of prolapsed hemorrhoids. The choice of nonsurgical therapy, however, should be tailored to the individual patient because RBL is associated with more pain whereas IRC may require additional treatment sessions for recurrence of symptoms. If possible, the patient should be allowed to choose between the two. Finally, direct comparison between hemorrhoidectomy and RBL indicates that hemorrhoidectomy is clearly more efficacious but is associated with significantly more pain and complications than RBL.

Based on a comprehensive review of the available evidence, therefore, it is my opinion that the optimal therapy for hemorrhoidal disease begins with fiber supplements. Short-term topical therapy to relieve symptoms has not been shown to be effective but carries little associated risk. If symptoms do not respond to conservative treatment, then either RBL or IRC is warranted. The choice of which treatment to pursue should be individualized, based on patient preference, once the risks and benefits of each have been explained. In either case, hemorrhoidectomy should be reserved for patients who fail nonoperative

therapy because of the greater risk of complications and postprocedure pain.

REFERENCES

1. Smith LE. Hemorrhoids. A review of current techniques and management. Gastroenterol Clin North Am 1987;16: 79–91.
2. Johanson JF, Sonnenberg A. The prevalence of hemorrhoids and chronic constipation. An epidemiologic study. Gastroenterology 1990;98:380–386.
3. Moesgaard F, Nielsen ML, Hansen JB, Knudsen JT. High-fiber diet reduces bleeding and pain in patients with hemorrhoids: A double-blind trial of Vi-Siblin. Dis Colon Rectum 1982;25:454–456.
4. Perez-Miranda M, Gomez-Cedenilla A, Leon-Colombo T, Pajares J, Jiminez J. Effect of fiber supplements on internal bleeding hemorrhoids. Hepatogastroenterology 1996;43: 1504–1507.
5. Broader JH, Gunn IF, Alexander-Williams J. Evaluation of bulk-forming evacuant in the management of haemorrhoids. Br J Surg 1974;61:142–144.
6. Webster DJT, Gough DCS, Craven JL. The use of bulk evacuant in patients with haemorrhoids. Br J Surg 1978;65: 291–292.
7. Cospite M. Double blind, placebo-controlled evaluation of clinical activity and safety of Daflon 500 mg in the treatment of acute hemorrhoids. Angiology 1994;45:566–573.
8. Godeberg P. Daflon 500 mg in the treatment of hemorrhoid disease: A demonstrated efficacy in comparison with placebo. Angiology 1994;45:574–578.
9. Thanapongsathorn W, Vajrabukka T. Clinical trial of oral diosmin (Daflon) in the treatment of hemorrhoids. Dis Colon Rectum 1992;35:1085–1088.
10. Gionchetti P, Campieri M, Belluzzi A, Brignola C, Miglioli M, Barbara L. 5-ASA suppositories in hemorrhoidal disease. Can J Gastroenterol 1992;6:18–20.
11. Dennison AR, Whitston RJ, Rooney S, Morris DL. The management of hemorrhoids. Am J Gastroenterol 1989;84: 475–481.
12. Weinstein SJ, Rypins EB, Houck J, Thrower S. Single session treatment for bleeding hemorrhoids. Surg Gynecol Obstet 1987;165:479–482.
13. Templeton JL, Spence RAJ, Kennedy TL, Parks TG, Mackenzie G, Hanna WA. Comparison of infrared coagulation and rubber band ligation for first and second degree haemorrhoids: A randomized, prospective clinical trial. Br Med J 1983;286:1387–1389.
14. Ambrose NS, Hares MM, Alexander-Williams J, Keighley MRB. Prospective, randomized comparison of photocoagulation and rubber band ligation in treatment of hemorrhoids. Br Med J 1983;286:1389–1391.
15. Walker AJ, Leicester RJ, Nicholls RJ, Mann CV. A prospective study of infrared coagulation, injection, and rubber band ligation in the treatment of hemorrhoids. Int J Colorectal Dis 1990;5:113–116.
16. Gartell PC, Sheridan RJ, McGinn FP. Outpatient treatment of hemorrhoids: A randomized clinical trial to compare rubber band ligation with phenol injection. Br J Surg 1985;72:478–479.
17. Sim AJW, Murie JA, Mackenzie I. Comparison of rubber band ligation and sclerosant injection for first and second degree haemorrhoids—A prospective clinical trial. Acta Chir Scand 1981;147:717–720.
18. Sim AJW, Murie JA, Mackenzie I. Three year follow-up study on the treatment of first and second degree hemorrhoids by sclerosant injection or rubber band ligation. Surg Gynecol Obstet 1983;157:534–536.
19. Ambrose NS, Morris D, Alexander-Williams J, Keighley MRB. A randomized trial of photocoagulation or injection sclerotherapy for the treatment of first- and second-degree hemorrhoids. Dis Colon Rectum 1985;28:238–240.
20. Johanson JF, Rimm A. Optimal nonsurgical treatment of hemorrhoids: A comparative analysis of infrared coagulation, rubber band ligation, and injection sclerotherapy. Am J Gastroenterol 1992;87:1600–1606.
21. Poen AC, Felt-Bersma RJ, Cuesta MA, Deville W, Meuwissen SG. A randomized controlled trial of rubber band ligation versus infrared coagulation in the treatment of internal hemorrhoids. Eur J Gastroenterol Hepatol 2000;12:535–539.
22. MacRae HM, McLeod RS. Comparison of hemorrhoidal treatment modalities. A meta-analysis. Dis Colon Rectum 1995;38:687–694.
23. MacRae HM, McLeod RS. Comparison of hemorrhoidal treatments: A meta-analysis. Can J Surg 1997;40:14–17.
24. Murie JA, Mackenzie I, Sim AJ. Rubber band ligation and hemorrhoidectomy for second and third degree hemorrhoids: A prospective clinical trial. Br J Surg 1980;67:786–788.
25. Lewis AA, Rogers HS, Leighton M. Trial of maximal anal dilatation, cryotherapy and elastic band ligation as alternatives to hemorrhoidectomy in the treatment of large prolapsing hemorrhoids. Br J Surg 1983;70:54–56.
26. Cheng FC, Shum DW, Ong GB. The treatment of second degree hemorrhoids by injection, rubber band ligation, maximal anal dilatation and hemorrhoidectomy, a prospective clinical trial. Aust N Z J Surg 1981;51:458–462.

Surgical Management of Hemorrhoids

Anthony J. Senagore, M.D.

There are few diseases more chronicled in human history than symptomatic hemorrhoidal disease.[1,2] References occur in ancient texts dating back to Babylonian, Egyptian, Greek, and Hebrew cultures.[1,2] Included in many of these writings are multiple recommended treatment regimens including anal dilatation, topical ointments, and the intimidating red hot poker.[3,4] Although few people have died of hemorrhoidal disease, many patients wish they had, particularly after therapy, and this fact led to the beatification of St. Fiachre, the patron saint of gardeners and hemorrhoid sufferers.[5] It is hoped that this discussion will guide the practitioner toward a more humane approach to the surgical management of hemorrhoidal disease, with an emphasis on cost-effectiveness with minimal morbidity and mortality.

ANATOMY/ETIOLOGY

The hemorrhoidal cushions appear predictably in the right anterior, right posterior, and left lateral positions, although there may be intervening secondary hemorrhoidal complexes, which blur this classic anatomy.[6] The blood supply is similarly constant, deriving from the superior rectal artery, a branch of the inferior mesenteric artery, the middle rectal arteries arising from the internal iliac arteries; and the inferior rectal arteries arising from the pudendal arteries. The venous drainage transitions from the portal venous system above the level of the dentate line to the systemic venous system below this level.[6]

The major anatomic distortion that must be addressed surgically is related to abnormalities within the connective tissue of these cushions, which produce prolapse of the hemorrhoidal tissue and dislocation of the anoderm.[7] This can occur as the result of excessive straining, chronic constipation, or low dietary fiber.[8] A clear understanding of the pathophysiology is important when considering therapeutic interventions. At the earlier stages of disease progression, when the major manifestation is transudation of blood through thin-walled damaged veins and/or arterioles, ablation of the vessels should be adequate. Conversely, in the late stages of the disease, when there is significant disruption of the mucosal suspensory ligament, relocation and fixation of the mucosa to the underlying muscular wall is required for effective therapy.[9]

EXCISIONAL HEMORRHOIDECTOMY

The decision to proceed to excisional hemorrhoidectomy requires a mutual decision by the physician and patient that medical and nonexcisional options have either failed or are not appropriate. The usual clinical symptoms that lead to surgical excision are frequent prolapsing of the internal hemorrhoids and anoderm, which results in discomfort and anal seepage. Alternatively, the thickened and prolapsing internal/external hemorrhoidal complexes may make anal hygiene difficult for the patient and may make excision preferable. The final indication for excisional hemorrhoidectomy, although this is debatable, is the development of acutely thrombosed and gangrenous internal hemorrhoids. Surgical excision of acutely thrombosed external hemorrhoids may also be warranted, primarily for more rapid pain relief and avoidance of a residual skin tag. Management of these external thromboses is usually easily managed in the office setting with local anesthesia and complete excision with or without skin closure.

Options for excisional hemorrhoidectomy include the following techniques: Milligan-Morgan hemorrhoidectomy; Ferguson closed hemorrhoidectomy; Whitehead hemorrhoidectomy; and the more recently described circular stapled hemorrhoidectomy. The procedures are usually performed in the operating theater, after minimal preoperative preparation of the bowel. The use of lasers for excisional hemorrhoidectomy offers no advantage and, in fact, results

From the Cleveland Clinic Foundation (A.J.S.), Cleveland, Ohio.
Address correspondence to: Dr. Anthony J. Senagore, Department of Surgery, Cleveland Clinic Foundation, 9500 Euclid Ave., A111, Cleveland, OH 44195. e-mail: senagoa@ccf.org

1091-255X/02/$—see front matter
PII: S1091-255X(01)00082-8

in delayed healing, increased pain, and increased cost.[10] The selection of anesthetic is usually left to the anesthesiologist and the patient; however, local anesthesia supplemented by the administration of intravenous narcotics and propofol is very effective and short acting. The use of spinal anesthesia, although effective, may increase the risk of postoperative urinary retention because of the increased intraoperative administration of intravenous fluids.

The Milligan-Morgan hemorrhoidectomy, which is widely performed in Europe, was originally described in 1937, and its efficacy has been subsequently documented in many series.[11–13] This technique includes resection of the entire enlarged internal hemorrhoid complex, ligation of the arterial pedicle, and preservation of the intervening anoderm.[10] The distal anoderm and external skin are left open to minimize the risk of infection in the wounds. Results of this technique have shown it to be a safe and effective means of managing advanced hemorrhoidal disease.[10] However, the fact that the external wounds are left open for delayed healing can be a cause of considerable discomfort and prolonged morbidity after this procedure.

The closed Ferguson hemorrhoidectomy was proposed as an alternative to the Milligan-Morgan technique and enjoys a similar large body of evidence regarding its safety and efficacy.[14–17] This technique employs an hourglass-shaped (centered at the midportion of the anoderm) excision of the entire internal/external hemorrhoidal complex, preservation of the internal and external anal sphincters, and primary closure of the entire wound. Occasionally it is necessary to undermine flaps of anoderm and perianal skin to allow removal of intermediate hemorrhoidal tissue, while preserving the bridges of anoderm between pedicles. This technical adjustment will avoid postoperative strictures.

The Whitehead hemorrhoidectomy, described in 1882, was devised to eradicate the enlarged internal hemorrhoidal tissue in a circumferential fashion and to relocate the prolapse dentate line, which is often a component of prolapsing hemorrhoids.[18] Although this technique enjoyed a long period of widespread application, it was subsequently largely abandoned because of the high rates of mucosal ectropion and anal stricture.[19–22] The technique is enjoying renewed support, with several authors documenting minimal stricture rates and no occurrences of mucosal ectropion.[23,24] Despite these promising reports, the Whitehead procedure is technically demanding because of the need to accurately identify the dentate line and relocate it to its proper position.

A new entry into the arena of excisional hemorrhoidectomy is the circular stapled hemorrhoidectomy.[25] The technique uses a circular, transanally placed pursestring suture, which is placed 4 cm from the dentate line and within the enlarged internal hemorrhoids. A 31 mm stapler is then placed transanally to perform a circumferential excision of the hemorrhoidal tissue and a repositioning and fixation of the anoderm to its proper location in the anal canal. The results appear promising with decreased postoperative pain, shorter periods of convalescence, and similar complication rates compared to other forms of excisional hemorrhoidectomy. There have been some concerns raised regarding protracted rectal pain after this approach and the potential for life-threatening infections, although larger series do not support these claims.

Regardless of the excisional technique used for treatment of advanced hemorrhoidal disease, the key to effective patient management is avoidance of postoperative complications. Pain is the most frequent complication and is the most feared sequela of the procedure from the patient's perspective. A variety of analgesic regimens have been recommended, usually consisting of a combination of oral and parenteral narcotics.[26–30] The use of local infiltration of bupivacaine into the wounds and perianal skin has been variably successful in long-term pain reduction.[31,32] Conversely, ketorolac has demonstrated considerable efficacy in managing posthemorrhoidectomy pain.[33] The use of alternative administration routes for narcotics, either by patch or subcutaneous pump, has been successful in controlling pain; however, the management of these routes of administration can be risky in the outpatient setting because of the risk of narcotic-induced respiratory depression. The most appropriate regimen after outpatient hemorrhoidectomy appears to be intraoperative use of ketorolac, sufficient doses of oral narcotic analgesics for home administration, and supplementation of the narcotics with an oral nonsteroidal medication.

Urinary retention is a frequent postoperative problem after hemorrhoidectomy, ranging in incidence from 1% to 52%.[34–37] A variety of strategies have been used to treat the problem, including parasympathomimetics, α-adrenergic blocking agents, and sitz baths.[38,39] The best approach, however, seems to be a strategy of prevention that includes limiting perioperative fluid administration to 250 ml, an anesthetic approach that avoids the use of spinal anesthesia, avoidance of anal packing, and an aggressive oral analgesic regimen.[40]

Early postoperative bleeding (<24 hours) occurs in approximately 1% of cases and represents a technical error requiring return to the operating theater for resuturing of the offending wound.[41] Delayed

hemorrhage of excisional hemorrhoidectomy occurs in 0.5% to 4% of cases at 5 to 10 days postoperatively.[42–44] The etiology has been held to be early separation of the ligated pedicle before adequate thrombosis in the feeding artery can occur.[45] The bleeding in this scenario is usually significant and requires some method for control of ongoing hemorrhage. Options include a return to the operating theater for suture ligation or tamponade at the beside by Foley catheter or anal packing.[46–47] The subsequent outcome after control of secondary hemorrhage is generally good, with virtually no risk of recurrent bleeding. It may be helpful to irrigate the distal colorectum with posthemorrhage enemas or at the time of intraoperative control of bleeding to avoid confusion when the residual clots pass per anum.

CONCLUSION

The management of symptomatic hemorrhoidal disease should be directed at the symptom complex of the individual patient. Most patients can be successfully treated by improving bowel function, correcting constipation, and the use of any of a variety of anal ointments. For persistent symptoms, either injection or banding of the internal hemorrhoids is predictably successful. Few patients should require excisional hemorrhoidectomy by any of the described techniques. Circular stapled hemorrhoidectomy may prove to be an effective, less painful technique to manage grade 3 hemorrhoids.

REFERENCES

1. Holley CJ. History of hemorrhoidal surgery. South Med J 1946;39:536.
2. Madoff RD. Biblical management of anorectal disease. Presented at the meeting of the Midwest Society of Colon and Rectal Surgeons. Breckenridge, CO, March 1991.
3. Dirckx JH. The biblical plague of "hemorrhoids." An outbreak of bilharziasis. Am J Dermatopathol 1985;7:341–346.
4. Maimonides M, Rosner F, Munter S. Treatise on Hemorrhoids. Philadelphia: JB Lippincott, 1969.
5. Rachochot JE, Petourand CH, Riovoire JO. Saint Fiacre: The healer of hemorrhoids and patron saint of proctology. Am J Proctol 1971;22:175–177.
6. Thompson WHF. The nature of haemorrhoids. Br J Surg 1975;62:542–552.
7. Morgado PJ, Suarez JA, Gomez LG, et al. Histoclinical basis for a new classification of hemorrhoidal disease. Dis Colon Rectum 1988;31:474–480.
8. Burkitt DP, Graham-Stewart CW. Hemorrhoids—Postulated pathogenesis and proposed prevention. Postgrad Med J 1975;51:631–636.
9. Haas PA, Fox TA, Haas GP. The pathogenesis of hemorrhoids. Dis Colon Rectum 1984;27:442–450.
10. Senagore A, Mazier WP, Luchtefeld MA, et al. The treatment of advanced hemorrhoidal disease: A prospective randomized comparison of cold scalpel vs. contact Nd:YAG laser. Dis Colon Rectum 1993;6:1042–1049.
11. Milligan ET, Morgan CN, Lond LE. Surgical anatomy of the anal canal, and the operative treatment of hemorrhoids. Lancet 1937;2:1119–1124.
12. Duhamel J, Romand-Heuer Y. Technische Besonderheiten bei der Hamorrhoidektomie nach Milligan und Morgan. Coloproctology 1980;4:265–266.
13. Tajana A. Hemorrhoidectomy according to Milligan-Morgan: Ligature and excision technique. Int Surg 1989;74: 158–161.
14. Ferguson JA, Heaton JR. Closed hemorrhoidectomy. Dis Colon Rectum 1959;2:176–179.
15. Muldoon JP. The completely closed hemorrhoidectomy: A reliable and trusted friend for 25 years. Dis Colon Rectum 1981;24:211–214.
16. McConnell JC, Khubchandani IT. Long-term follow-up of closed hemorrhoidectomy. Dis Colon Rectum 1983;26: 797–799.
17. Ganchrow MJ, Mazier WP, Friend WG, Ferguson JA. Hemorrhoidectomy revisited: A computer analysis of 2,038 cases. Dis Colon Rectum 1971;14:128–133.
18. Whitehead W. The surgical treatment of hemorrhoids. Br Med J (Clin Res) 1882;1:148–150.
19. Andrews E. Disastrous results following Whitehead's operation and the so-called American operation. Columbus Med J 1895;15:97–106.
20. Andrews E. Some of the evils caused by Whitehead's operation and by its modification, the American operation. Trans Ill Med Soc 1895:433–446.
21. Khubchandani M. Results of Whitehead operation. Dis Colon Rectum 1984;27:730–732.
22. Rand AA. The sliding skin-flap graft operation for hemorrhoids: A modification of the Whitehead procedure. Dis Colon Rectum 1969;12:265–276.
23. Wolff BG, Culp CE. The Whitehead hemorrhoidectomy. Dis Colon Rectum 1988;31:587–590.
24. Bonello JC. Who's afraid of the dentate line? The Whitehead hemorrhoidectomy. Am J Surg 1988;156:182–186.
25. Kohlstadt CM, Weber J, Prohm P. Stapler hemorrhoidectomy. A new alternative to conventional methods. Zentralbl Chir 1999;124:238–243.
26. O'Donovan S, Ferrara A, Larach S, Williamson P. Intraoperative use of Toradol facilitates outpatient hemorrhoidectomy. Dis Colon Rectum 1994;37:793–799.
27. Kuo RJ. Epidural morphine for post-hemorrhoidectomy analgesia. Dis Colon Rectum 1984;27:529–530.
28. Kilbride MJ, Senagore AJ, Morse M. Improving patient safety with transdermal fentanyl for post-hemorrhoidectomy pain (letter). Dis Colon Rectum 1994;104.
29. Kilbride MJ, Morse M, Senagore AJ. Transdermal fentanyl improves management of postoperative hemorrhoidectomy pain. Dis Colon Rectum 1994;37:1070–1072.
30. Goldstein ET, Williamson PR, Larach SW. Subcutaneous morphine pump for postoperative hemorrhoidectomy pain management. Dis Colon Rectum 1993;36:439–446.
31. Hussein MK, Taha AM, Haddad FF, Bassim YR. Bupivacaine local injection in anorectal surgery. Int Surg 1998; 83:56–57.
32. Chester JF, Stanford J, Gazet JC. Analgesic benefit of locally injected bupivacaine after hemorrhoidectomy. Dis Colon Rectum 1990;33:487–489.
33. Bleday R, Pena PJ, Rothenberger DA, Goldberg SM, Buls JG. Symptomatic hemorrhoids: Current incidence and complications of operative therapy. Dis Colon Rectum 1992;35:477–481.
34. Hoff SD, Bailey HR, Butts DR, et al. Ambulatory surgical

hemorrhoidectomy—A solution to postoperative urinary retention? Dis Colon Rectum 1994;37:1242–1244.
35. Petros JG, Bradley TM. Factors influencing postoperative urinary retention in patients undergoing surgery for benign anorectal disease. Am J Surg 1990;159:374–376.
36. Tammela T, Kontturi M, Lukkarinen O. Postoperative urinary retention. I. Incidence and predisposing factors. Scand J Urol Nephrol 1986;20:197–201.
37. Leventhal A, Pfau A. Pharmacologic management of postoperative over-distension of the bladder. Surg Gynecol Obstet 1976;146:347–348.
38. Shafik A. Role of warm water bath in inducing micturition in postoperative urinary retention after anorectal operations. Urol Int 1993;50:213–217.
39. Hoff SD, Bailey HR, Butts DR, Max E, Smith KW, Zamora LF, Skakun GB. Ambulatory surgical hemorrhoidectomy—A solution to postoperative urinary retention? Dis Colon Rectum 1994;37:1242–1244.
40. Corman ML. Complications of hemorrhoid and fissure surgery. In Ferrari BT, Ray JE, Gathright JB, eds. Complications of Colon and Rectal Surgery—Prevention and Management. Philadelphia: WB Saunders, 1985, pp 91–100.
41. Kilbourne NJ. Internal hemorrhoids: Comparative value of treatment by operative and by injection methods—A survey of 62,910 cases. Ann Surg 1934;99:600–608.
42. Salvati EP, Eisenstat TE. Hemorrhoidal disease. In Zuidema GD, Condon RE, eds. Shackelford's Surgery of the Alimentary Tract, 3rd ed. Philadelphia: WB Saunders, 1991, pp 294–307.
43. Milsom JW. Hemorrhoidal disease. In Wexner SD, Beck DE, eds. Fundamentals of Anorectal Surgery. New York: McGraw Hill, 1992, pp 192–214.
44. Gabriel WB. Haemorrhoids. In: The Principles and Practice of Rectal Surgery, 5th ed. Springfield, Ill.: Charles C Thomas, 1963, pp 110–164.
45. Rosen L, Sipe P, Stasik JJ, Riether RD, Trimpi HD. Outcome of delayed hemorrhage following surgical hemorrhoidectomy. Dis Colon Rectum 1993;36:743–746.
46. Cirocco WC, Golub RW. Local epinephrine injection as treatment for delayed hemorrhage after hemorrhoidectomy. Surgery 1995;117:235–237.
47. Basso L, Pescatori M. Outcome of delayed hemorrhage following surgical hemorrhoidectomy. Dis Colon Rectum 1994;37:288–289.

Advanced Technology in the Management of Hemorrhoids: Stapling, Laser, Harmonic Scalpel, and Ligasure

James Fleshman, M.D.

LASER

The use of laser technology for the treatment of hemorrhoids continues to be controversial. The theoretical advantages of lasers have included reduced pain and improved healing. The use of laser for the treatment of hemorrhoids has been the impetus for the development of specialty clinics and has served as an excellent marketing tool for those surgeons focusing primarily on treating hemorrhoids. The evidence for this modality as the preferred energy source is scarce.

A randomized controlled trial comparing closed Ferguson hemorrhoidectomy with scissors or laser was reported by Wang et al.[1] in 1991. A poorly matched group of 88 patients was treated with excisional hemorrhoidectomy using scissors or laser to perform the excision. The wounds were closed with running absorbable sutures. The laser-treated group was reported to experience less pain and a shorter length of hospital stay. However, healing time was approximately 2 weeks longer for the laser-treated group.

Senagore et al.,[2] in 1993, reported the results of a randomized controlled trial comparing Nd:YAG laser and cold scalpel to perform closed Ferguson hemorrhoidectomy. The patients were matched to have grade 3 internal mixed hemorrhoids. The 81 patients were found to have no difference in pain based on a visual analogue pain scale. The cost of laser hemorrhoidectomy was, on average, $480 more per case. There was also an increase in wound dehiscence in the laser-treated group. Patients were treated as outpatients so the advantage of no hospital stay was noted.

Even though randomized controlled data suggest no advantage for laser in hemorrhoidectomy, reports continue to appear in the literature. Zahir et al.[3] recently reported on a retrospective review of a group of 50 patients divided between laser and standard hemorrhoidectomy. The laser-treated group reported pain to be 65% less than that in the group treated with standard hemorrhoidectomy at 1 week; the laser-treated group also had painless defecation by 5 days, less drainage, and returned to work twice as quickly as patients undergoing standard hemorrhoidectomy. Hospital and surgical costs were also lower for the laser-treated group. These findings will need to be confirmed in a randomized controlled trial.

HARMONIC SCALPEL/LIGASURE

Newer energy sources such as harmonic scalpel and Ligasure (U.S. Surgical, Norwalk, Connecticut) have not been evaluated thoroughly. Each of these modalities relies on energy to ligate the hemorrhoidal vessels rather than a suture placed at the vascular pedicle. Theoretically this may reduce anal sphincter spasm and pain.

Armstrong[4] presented preliminary data at the 2000 meeting of the American Society of Colorectal Surgeons (in poster form) from a randomized comparison of the use of electrocautery and harmonic scalpel for Ferguson hemorrhoidectomy in grade 3 and 4 disease. He found that patients noted less pain on an analogue scale and needed fewer narcotics when the harmonic scalpel was used. The harmonic scalpel system vibrates at 55,000 Hz and results in coagulation of blood and fusion of vessel walls. However, complications and recurrence are not mentioned. There was no difference in the length of hospital stay.

The Ligasure (bipolar cautery with automatic "off" switch when impedance reaches a critical level) has been used when treating hemorrhoids to control the vascular pedicle. There are currently no reports available regarding this technique. Controlled trials

From the Division of Colon and Rectal Surgery (J.F.), Washington University School of Medicine, St. Louis, Missouri.
Address correspondence to: Dr. James Fleshman, Department of Surgery, Division of Colorectal Surgery, 216 S. Kingshighway, St. Louis, MO 63110-1026.

1091-255X/02/$—see front matter
PII: S1091-255X(01)00083-X

are needed to evaluate these modalities because of the potential added cost to the procedure.

CIRCULAR STAPLED HEMORRHOIDECTOMY

The most significant change in hemorrhoid surgery may be the use of a modified circular stapler to resuspend the prolapsing mucosa and vascular cushions and remove any redundant distal rectal mucosa. This technique was initially reported by Dr. Don Peck, and modified and standardized by Dr. Antonio Longo. Large prospective series of patients treated with circular stapled hemorrhoidectomy (CSH) for grade 3 hemorrhoids have been accumulated in Europe and the Middle East.[5] Patients treated with CSH reportedly have less pain and excellent control of symptoms. However, complications have included rectal perforation and pelvic sepsis.[6] The technique is simple but has some specific features that guarantee its success. These include placement of the purse-string sutures for the circular stapler, insertion and removal of the stapler, and ligation of vascular pedicles at the staple line for control of hemorrhage.

The controversy surrounding this technique arises from an inability to classify hemorrhoids objectively. There is no good standard classification system that can be used to guarantee that all patients entered into trials of this technique have the same extent of hemorrhoidal disease. As a result, we are unable to determine whether patients in existing trials or series might have been treated with a less invasive and less expensive approach such as rubber band ligation. This is extremely important, because the number of hemorrhoidectomies performed in Europe using this technique far exceeds the number of excisional hemorrhoidectomies performed in the United States over the same period of time.

The first report on the "Longo" CSH in the English literature was by O'Bichere et al.[7] in 1998. The technique was shown to be feasible as an outpatient treatment of hemorrhoids. Thus far, CSH has only been compared to Milligan-Morgan (open) hemorrhoidectomy. Mehigan et al.[8] reported a randomized controlled trial of 40 patients comparing the Milligan-Morgan procedure to CSH.[8] Pain scales, interviews, and maximum 10-week follow-up revealed equivalent operative times, lengths of stay, complications, symptom control, and short-term functional outcome for both groups. These investigators recommended long-term follow-up to fully evaluate the technique. Rowsell et al.[9] found similar results in a smaller randomized trial of 22 patients but once again had no long-term follow-up.

The largest randomized controlled trial thus far was conducted by Ho et al.[10] in Singapore. A group of 119 patients were randomized to the Milligan-Morgan procedure or CSH for grade 3 and 4 hemorrhoids. Postoperative incontinence, sphincter injury, complications, length of hospital stay, and operative times were equivalent. Postoperative pain, narcotic use, bleeding, healing, and return to work were better in the CSH group. Cost was 30% higher with CSH.

A recent report by Cheetham et al.[11] at St. Marks Colorectal Unit revealed a significant incidence of persistent pain and fecal urgency (31%) after stapled hemorrhoidectomy. This was attributed to muscle being incorporated into the mucosal "doughnut" and probably indictates placement of the pursestring sutures within the anal canal itself rather than in the distal rectum. The randomized trial was discontinued as a result of these findings.

A pilot group of patients has been studied at the University of Illinois and Washington University, and the findings were reported at the 2001 meeting of the American Society of Colorectal Surgeons. Patients seem to have less pain and tolerate the procedure on an outpatient basis. The indications seem limited to those individuals with hemorrhoids that have not or will not respond to rubber band ligation unless a combination procedure (CSH and excision of the external component) can be shown to be as cost-effective as excision alone for nonreducible grade 4 hemorrhoids. A randomized controlled trial is currently in the beginning stages, with pain, complications, cost, and recurrence as the end points.

REFERENCES

1. Wang JY, Chang-Chien CR, Chen J-S, Lai C-R, Tang R. The role of lasers in hemorrhoidectomy. Dis Colon Rectum 1991;34:78–82.
2. Senagore A, Mozier WP, Luchtefeld MA, MacKeigan JM, Wengert T. Treatment of advanced hemorrhoidal disease: A prospective randomized comparison of cold scalpel vs contact Nd:YAG laser. Dis Colon Rectum 1993;36:1042–1049.
3. Zahir KS, Edwards RE, Vecchia A, Dudrick SJ, Tripodi G. Use of the Nd-YAG laser improves quality of life and economic factors in the treatment of hemorrhoids. Conn Med 2000;64:199–203.
4. Armstrong D. Randomized controlled trials of harmonic scalpel and electrocautery for hemorrhoidectomy [abstr]. Presented at the American Society of Colorectal Surgeons, Boston, MA, June 2000.
5. Levanon A, Biterman A, Behar A, Cohen O. Hemorrhoidectomy using a circular stapler. Harefuah 2000;138:12–14.
6. Molloy RG, Kingsmore D. Life threatening pelvic sepsis after stapled haemorrhoidectomy [letter]. Lancet 2000;355:810.
7. O'Bichere A, Laniado M, Sellu D. Stapled haemorrhoidectomy: A feasible day-case procedure. Br J Surg 1998;85:377–378.

8. Mehigan BJ, Monson JR, Hartley JE. Stapling procedure for haemorrhoids versus Milligan-Morgan haemorrhoidectomy: Randomised controlled trial. Lancet 2000;355:782–785.
9. Rowsell M, Bello M, Hemingway DM. Circumferential mucosectomy (stapled haemorrhoidectomy) versus conventional haemorrhoidectomy: Randomised controlled trial. Lancet 2000;355:779–781.
10. Ho YH, Cheong WK, Tsang C, Ho J, Eu KW, Tang CL, Seow-Choen F. Stapled hemorrhoidectomy: Cost and effectiveness. Randomized controlled trial including incontinence scoring, anorectal manometry and endoanal ultrasound assessments at up to 3 months. Dis Colon Rectum 2000;43:1666–1675.
11. Cheetham JM, Mortensen NJ, Nystrom PO, Kamm MA, Phillips RK. Persistent pain and fecal urgency after stapled hemorrhoidectomy. Lancet 2000;356:730–733.

2001 Consensus Statement on Benign Anorectal Disease

W. Scott Helton, M.D., for The SSAT, AGA, ASGE Consensus Panel

CHRONIC ANAL FISSURE

The prevalence of anal fissure is approximately one fifth that of symptomatic hemorrhoids. Most anal fissures result from increased tone in the internal anal sphincter combined with relative ischemia in the posterior midline anal canal. Successful treatment for these chronic anal fissures requires reduction in anal canal resting pressure. This can be accomplished by inducing smooth muscle relaxation of the internal anal sphincter pharmacologically or surgically. Pharmacologic options include the topical application of nitric oxide donors (glyceryl trinitrate [GTN], isosorbide dinitrate), calcium channel antagonists (diltiazem cream), anticholinergics (bethanechol cream), or the injection of botulinum toxin. The optimal dose of GTN is a pea-sized volume of a 0.2% formulation applied three times a day. Higher concentrations are associated with increased side effects with no additional benefit. Healing occurs in one third to two thirds of patients with GTN therapy; a rate superior to that of placebo. After healing, fissures recur in approximately one half of patients. Headaches occur in a significant number of patients. Patients with orthostatic hypotension, intolerance to nitrates, and ischemic heart disease are probably best managed with an alternative therapy. In one clinical trial, botulinum toxin was reported to be superior to GTN in healing chronic anal fissures, and with a lower rate of complications. Preliminary evidence suggests that healing with diltiazem and bethanechol gel is comparable to healing with GTN with no identified side effects.

Surgical options for chronic anal fissure include anal stretch, open and closed lateral sphincterotomy, and posterior sphincterotomy. Lateral sphincterotomy is superior to GTN in healing chronic anal fissures. Anal stretch should be abandoned because of a higher risk of anal incontinence and a lower rate of healing when compared to sphincterotomy. Open or closed lateral sphincterotomy is the preferred surgical treatment of anal fissure and is associated with a small but definite risk of flatus incontinence.

At present, the best reported results of pharmacologic treatment for anal fissure are achieved with botulinum toxin. However, these results are from only one trial with limited follow-up, and there are significant concerns regarding the safety, cost, and convenience of botulinum toxin. Currently, the available data suggest that the first-line treatment is GTN. However, the side effect profile limits its usefulness, making newer topical agents potentially more attractive. Pharmacologic treatment should balance efficacy, short- and long-term side effects, convenience, and expense. These data will permit the informed patient to make a choice.

HEMORRHOIDS

Symptomatic hemorrhoids are a common medical condition occurring in 42 per 1000 of the United States population, or 10,600,000 Americans per year. It is estimated that two and a half million persons per year visit physicians for treatment of hemorrhoids; 160,000 have operations in short-stay hospitals and 959,000 hospital days are used by patients with hemorrhoids.[1]

Initial treatment of hemorrhoid disease typically begins with fiber supplements and increased water intake. Short-term topical therapy to relieve symptoms has not been shown to be effective in randomized clinical trials but carries little associated risk. If symptoms do not respond to conservative treatment, rubber band ligation or infrared coagulation is warranted. The choice of which treatment to pursue should be individualized based on patient preference and operator experience. Hemorrhoidectomy should

From the Division of General Surgery (W.S.H.), University of Illinois, Chicago, Illinois.
Address correspondence to: Dr. W. S. Helton, University of Illinois, Division of General Surgery (MC 958), 840 South Wood St., Room 435E CSB, Chicago, IL 60612-7233. e-mail: scoth@uic.edu

Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(01)00084-1

be reserved for patients in whom nonoperative therapy is unsuccessful, because of the increased risk of complications, postprocedure pain, and cost of surgical treatment. Indications for hemorrhoidectomy are frequent symptomatic prolapse of internal hemorrhoids and anoderm, which results in discomfort and anal seepage or the appearance of external hemorrhoids.

Open (Milligan-Morgan) and closed (Ferguson) hemorrhoidectomies have similar safety and efficacy, and are the preferred surgical approaches for hemorrhoids; there is insufficient evidence to recommend one excision technique over the other. Surgical treatment of internal hemorrhoids using a circular stapler may result in less pain and a shorter recovery period, although the long-term efficacy of this technique remains to be defined. The use of lasers and other energy-based excision techniques for excision hemorrhoidectomy offers no advantage over traditional hemorrhoidectomy and causes delayed healing, increased pain, and increased cost. Anal stretch and lateral sphincterotomy should be abandoned because of the high risk of early and late anal incontinence and no reduction in postoperative pain. Pain is the most common side effect after hemorrhoidectomy and is best managed by the use of intraoperative ketorolac in combination with oral narcotic analgesics and an oral nonsteroidal medication. Evidence also indicates that patients should begin taking laxatives at least 2 days before the operation. Postoperative oral metronidazole may reduce postoperative pain after open hemorrhoidectomy.

AREAS FOR FUTURE CLINICAL INVESTIGATION

Controlled clinical trials are warranted to establish optimal medical therapy for anal fissure, and are needed to define cost-effectiveness and patient satisfaction. In these trials, patients should be equally matched with respect to anatomic features of anal fissure and signs of chronicity, and follow-up should be at least 1 year. Prospective randomized clinical trials are warranted to establish the cost-effectiveness of circular stapling vs. traditional excisional hemorrhoidectomy. A randomized clinical trial testing the efficacy of chemical sphincterotomy combined with surgery as an adjunct for pain reduction is also warranted.

REFERENCES

1. Johanson JF. Hemorrhoids. In: Everhart JE. Ed. Digestive Diseases in the United States: Epidemiology and impact. US Department of Health and Human Services. Public Health Service, National Institutes of Health, National Institutes of Diabetes, Digestive and Kidney Diseases. Washington, D.C.: US Government Printing Office. 1994. NIH Publication no. 94-1447, pp 271–298.

Induction of Cyclooxygenase-2 and Invasiveness by Transforming Growth Factor-β_1 in Immortalized Mouse Colonocytes Expressing Oncogenic Ras

Christopher D. Roman, M.D., Jason Morrow, M.D., Robert Whitehead, Ph.D., R. Daniel Beauchamp, M.D.

Cyclooxygenase-2 (COX-2) expression appears to be important in colorectal carcinogenesis. Elevated COX-2 expression and activity have been observed in several different transformed cell types. Prior studies implicating involvement of the Ras oncogene and growth factors on COX-2 expression were largely derived from rat small intestinal cell lines. We have investigated whether mouse colonocyte COX-2 levels are regulated by oncogenic Ras or transforming growth factor-β_1 (TGF-β_1), and whether these factors also serve to regulate cellular invasiveness. Young adult mouse colonocyte cells are colonocytes derived from the "Immortomouse" and immortalized by the SV40 large T antigen. Young adult mouse colonocyte Ras cells were derived by transfection of young adult mouse colonocyte cells with oncogenic Ha-Ras and are known to be tumorigenic. We found that the induction of COX-2 and eicosanoid release were augmented in the presence of activated Ras and that TGF-β_1 caused a further increase in COX-2 in the Ras-transformed mouse colonocytes. Increased COX-2 expression was correlated with increased release of prostaglandins E_2 and I_2. Activated Ras and TGF-β increased the invasiveness of the young adult mouse colonocyte cells, but treatment with a COX-2 inhibitor did not inhibit invasiveness. Thus we found that transforming growth factor-β collaborates to increase COX-2 expression, protaglandin release, and invasiveness in mouse colonocytes, but the increased COX-2 activity does not appear to contribute to the invasive response. (J GASTROINTEST SURG 2002;6:304–309.)

KEY WORDS: Cyclooxygenase-2, carcinogenesis, Immortomouse, transforming growth factor-β

Colorectal cancer is the second most common fatal malignancy in the Western world, with approximately 150,000 new cases accounting for 55,000 deaths in the United States every year.[1] A growing body of evidence suggests that cyclooxygenase activity and the subsequent synthesis of prostaglandins are involved in colorectal carcinogenesis. Cyclooxygenase-2 (COX-2) expression has been shown to modulate production of angiogenic factors that stimulate endothelial migration by colon cancer cells.[2] COX-2 expression in intestinal epithelial cells also enables them to resist apoptosis.[3] The involvement of cyclooxygenase in tumor production is supported by the antineoplastic effects of inhibitors of this enzyme such as nonsteroidal anti-inflammatory drugs (NSAIDs). Early studies in animal models of colon cancer indicated that NSAIDs could inhibit intestinal tumor formation after carcinogen exposure.[4,5] Recent studies have shown a 40% to 50% decrease in the risk of colorectal carcinoma in patients who use aspirin regularly.[6–8]

In 1991, patients with the hereditary colon cancer syndrome referred to as familial adenomatous polyposis coli (FAP) were treated with sulindac and exhibited a significant repression of polyps. Carefully designed follow-up studies have also shown a significant reduction in polyp size and number in patients with FAP who are treated with sulindac.[9–11] In addition, NSAIDs have been shown to be effective in reducing tumor burden and growth in mouse models

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (poster presentation).

From the Departments of Surgery (C.D.R., R.D.B.), Cell Biology (R.D.B.), and Medicine (J.M., R.W.), Vanderbilt University Medical Center, Vanderbilt–Ingram Cancer Center, Nashville, Tennessee.

Supported by National Institutes of Health grants CA69457, CA77839, and DK52334 (R.D.B.), and by an American College of Surgeons Resident Research Scholarship (C.D.R.).

Reprint requests: R. Daniel Beauchamp, M.D., Department of Surgery, Vanderbilt University Medical Center, D5230 MCN, 21st Avenue South, Nashville, TN 37232-2730. e-mail: daniel.beauchamp@surgery.mc.vanderbilt.edu

Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(01)00041-5

of FAP[12] and in xenograft models of human cancer in nude mice.[13]

The ability of various cytokines, growth factors, oncogenes, and tumor suppressors to induce COX-2 expression has been demonstrated in several cell types.[14–21] The transforming growth factor–beta (TGF-β) family comprises a large number of structurally related polypeptide growth factors, and each of these is capable of regulating a complex array of cellular processes.[22–24] These processes include cell proliferation, differentiation, motility, adhesion, and death. Although TGF-β plays a primary role as a growth inhibitor and potential tumor suppressor, there is increasing evidence of its ability to promote tumorigenesis and contribute to the metastatic phenotype of tumor cells. Increased expression of TGF-β is associated with worse outcome in colorectal cancer.[25] Loss of responsiveness to the growth inhibitory effects of TGF-β occurs in many types of cancer cells including pancreatic,[26] breast,[27,28] and colorectal[29] cancer cells.

Mutational activation of a Ras oncogene is one of the most common genetic lesions in human cancer. Approximately 50% of human colorectal tumors have an activating k-Ras mutation.[30,31] Intestinal cells transformed by oncogenic Ras are resistant to the growth inhibitory effects of TGF-β.[32,33] Activation of Ras GTPase and TGF-β in rat small intestinal epithelial cells (RIE-1) each causes modest increases in COX-2 expression, but simultaneous activation of Ras signaling and TGF-β receptor activation results in a synergistic increase in COX-2 expression.[14]

Prior studies implicating involvement of the Ras oncogene and growth factors on COX-2 expression were largely derived from rat small intestinal cell lines as opposed to colonic epithelial cells. The purpose of this study was to determine whether mouse colonocyte COX-2 levels are regulated by oncogenic Ras and TGF-β_1, and whether these factors regulate cellular invasiveness.

MATERIAL AND METHODS

Cultured Cells

Cells used in these experiments consisted of YAMC (Young Adult Mouse Colonocyte) and YAMC-Ras cell lines. These colonocytes are derived from the "Immortomouse" and are conditionally immortalized by the presence of an SV40 large T antigen. This is temperature-sensitive antigen that allows these cells to grow indefinitely at 33° C. They also contain a γ-interferon promoter that serves to help stimulate cellular growth. YAMC-Ras cells were derived by stable transfection of YAMC cells with oncogenic Ha-Ras and are known to be tumorigenic.[34] Cell were grown in William's medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 100 units γ-interferon/L.

Western Blot Analysis

Cells were treated with TGF-β_1 (5 ng/ml) after 24 hours of serum starvation. Cells were lysed at 0, 8, and 24-hour intervals using NP-40 lysis buffer containing 50 mmol/L Tris-Cl, pH 7.8, 150 mmol/L NaCl, 1% nonidet P-40, 10 mmol/L EDTA, 10 mg/ml phenylmethylsulphonyl fluoride, 20 μg/ml FOY-305 protease inhibitor (Ono Pharmaceutical Co., Osaka, Japan), 1 mmol/L sodium orthovanadate, and 5 mmol/L sodium fluoride. Protein was subjected to Western blot analysis with the use of COX-2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and antigoat COX-2 antibody (Santa Cruz Biotechnology).

Invasion Assays

The effect of TGF-β_1 on cell invasiveness was examined in Boyden chambers with type I collagen–coated transwell inserts. Approximately 100 μl type I collagen was placed in the insert 24 hours before the experiment. Cells were seeded (1×10^4) into the transwell using William's medium containing 0.2% fetal bovine serum. The lower well contained 5 ng/ml TGF-β_1 with and without the COX-2 inhibitor NS-398 (Cayman Chemical Co., Ann Arbor, MI), also in 0.2% medium, and was allowed to incubate for 24 hours. After 24 hours, the inserts were fixed and stained to evaluate the extent of cellular invasion.

Quantification of Eicosanoids

Subconfluent cell cultures were established and the cells were treated with TGF-β_1 in serum-free media followed by the addition of arachidonic acid (15 μm) before each time point. This was repeated after 24 hours of serum starvation. Media were collected at 0, 2, and 8 hours. The prostaglandin (PGE_2 and PGI_2) formation in the medium was quantified by means of stable isotope dilution techniques employing gas chromatography negative ion chemical ionization mass spectrometry. The results are expressed as nanograms of PGE_2 and PGI_2 per milliliter of medium. COX-2 inhibition was again achieved with the use of NS-398.

RESULTS

As expected, the YAMC-Ras cells expressed higher levels of p21 H-Ras protein (Fig. 1, *A*). The

effect of TGF-β on COX-2 expression was evaluated in YAMC and YAMC-Ras cells. Under basal conditions, YAMC-Ras cells expressed a higher level of COX-2 in comparison to the parental YAMC cells. Treatment with TGF-β_1 (5 ng/ml) after serum starvation resulted in a slight increase in COX-2 expression in YAMC cells and a more marked increase in YAMC-Ras cells (Fig. 1, *B*).

COX-2 is a key enzyme required for prostaglandin synthesis; therefore release of PGE_2 and PGI_2 were evaluated in YAMC and YAMC-Ras cells in the presence and absence of TGF-β_1 (5 ng/ml). Prostaglandin (PGE_2 and PGI_2) synthesis and release were evaluated in the presence of an excess of arachidonic acid (15 μm). PGE_2 and PGI_2 release were significantly increased in both cell lines by TGF-β_1 (Figs. 2 and 3). There was a 1.5-fold increase in PGI_2 release and a 2.0-fold increase in PGE_2 release in the YAMC cells after TGF-β treatment. After similar treatment in the YAMC-Ras cells, there was a more pronounced 2.0-fold increase in PGI_2 release and a 4.0-fold increase in PGE_2 release.

The effect of oncogenic Ras and TGF-β treatment on colonocyte invasiveness was examined using a modified Boyden chamber assay. The YAMC-Ras cells exhibited 4.6-fold greater invasiveness as compared to the YAMC cells. YAMC cell invasion was increased by 30% after TGF-β_1 treatment. In contrast, TGF-β_1 increased the number of invasive YAMC-Ras cells by 2.4-fold (Fig. 4).

To determine whether COX-2 activity was necessary for the increased invasiveness, the experiment was repeated in the presence of the COX-2–specific inhibitor NS-398 at doses in excess of that required for maximal inhibition of prostaglandin synthesis.[35] These doses of NS-398 were sufficient to achieve a greater than 95% reduction in prostaglandin release. The average serum concentration of PGI_2 after 8-hour treatment of the YAMC-Ras cells with TGF-β_1 alone was 21 ng/ml; this was decreased to less than 0.5 ng/ml after the addition of NS-398 ($P < 0.0001$). Similarly, in YAMC-cells, this concentration was decreased from 10 ng/ml to less than 0.05 ng/ml ($P < 0.0001$). NS-398 had no effect on TGF-β–induced invasiveness (Fig. 5).

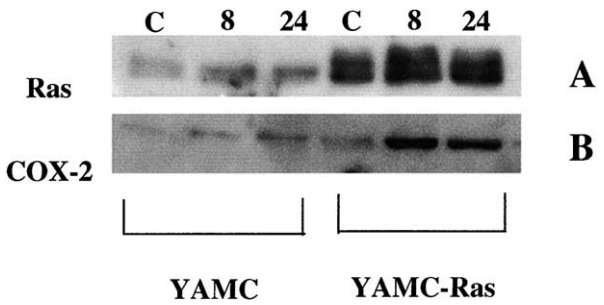


Fig. 1. Effect of TGF-β_1 on COX-2 and Ras expression in YAMC and YAMC-Ras cells. **A**, Ha-Ras expression was analyzed after the addition of TGF-β_1 (5 ng/ml). Lysates were collected at 0, 8, and 24 hours, and 50 μg samples were analyzed by Western blot analysis. **B**, COX-2 expression was analyzed after the addition of TGF-β_1 (5 ng/ml). Lysates were collected at 0, 8, and 24 hours, and 50 μg samples were analyzed by Western blot analysis.

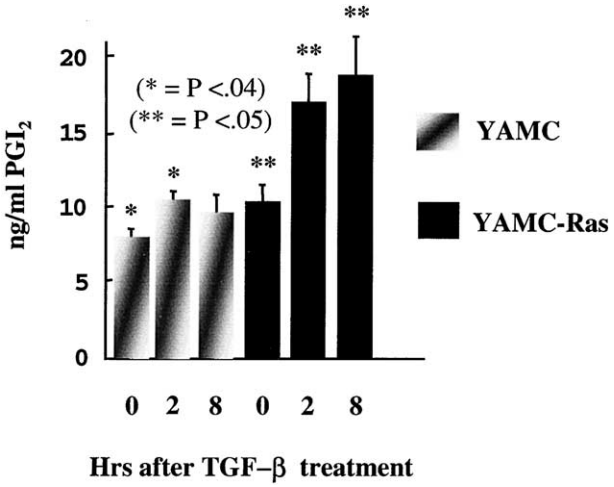


Fig. 2. PGI_2 release in YAMC and YAMC-Ras cells after TGF-β_1 treatment. Cells were treated with TGF-β_1 (5 ng/ml), and media were collected and analyzed by gas chromatography and mass spectrometry at 0, 2, and 8 hours. Arachidonic acid (15 μm) was added 20 minutes before media collection.

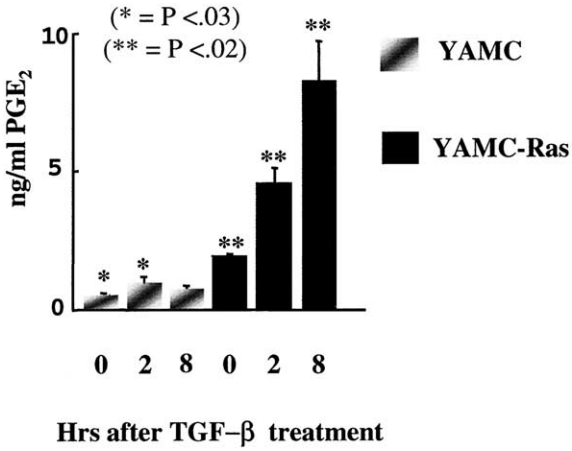


Fig. 3. PGE_2 release in YAMC and YAMC-Ras cells after TGF-β_1 treatment. Cells were treated with TGF-β_1 (5 ng/ml), and media were collected and analyzed by gas chromatography and mass spectrometry at 0, 2, and 8 hours. Arachidonic acid (15 μm) was added 20 minutes before media collection.

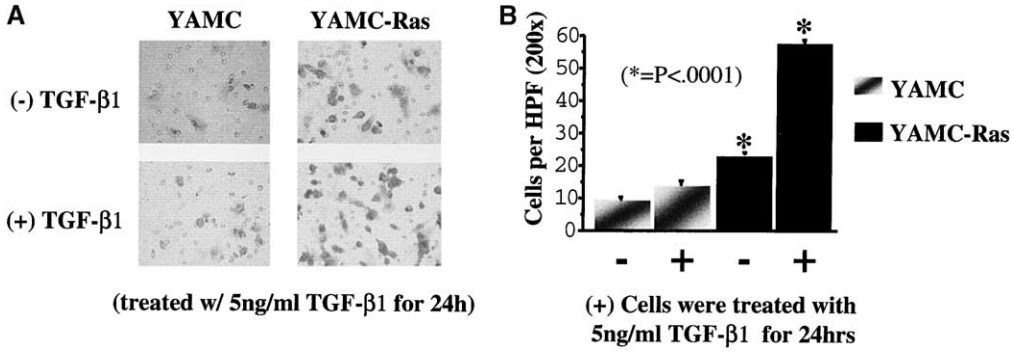


Fig. 4. Boyden chamber invasion assay analysis of YAMC and YAMC-Ras cells after TGF-β_1 treatment. YAMC and YAMC-Ras cells (1×10^4) were seeded onto type 1 collagen gel and tretaed with TGF-β_1 (5 ng/ml) for 24 hours. Membranes were preserved and cells were fixed and stained with methyl alcohol and crystal violet. **A,** High-power (×200) microscopic view of membranes. **B**, Cell counts per high-power field of YAMC and YAMC-Ras cells after membrane preservation.

DISCUSSION

In these experiments we have found that the induction of COX-2 and eicosanoid release were augmented in the presence of activated Ras and that TGF-β_1 caused a further increase in COX-2 in the Ras-transformed mouse colonocytes. The increase in COX-2 expression correlated with increased release of PGE_2 and PGI_2. Activated Ras and TGF-β each contributed to increased invasiveness in the colonocytes, but treatment with a COX-2 inhibitor did not prevent invasiveness in either YAMC or YAMC-Ras cells exposed to TGF-β. Thus we found that TGF-β collaborates to increase COX-2 expression, prostaglandin release, and invasiveness in mouse colonocytes, but increased COX-2 activity and prostaglandin synthesis do not appear to contribute to the invasive response.

Although the expression of COX-2 is often associated with neoplastic transformation, the precise role of COX-2 in this transformation is unclear. We have previously reported that treatment of RIE cells with TGF-β causes transient induction of COX-2 and increased prostaglandin production in small intestinal epithelial cells.[36–38] COX-2 mRNA and protein are markedly increased along with an increase in prostacyclin in RIE cells (RIE-TR) transformed by prolonged treatment with TGF-β_1.[39] Interestingly, the RIE-TR cells are refractory to growth inhibitory effects of TGF-β but retain TGF-β–dependent COX-2 overexpression. COX-2 overexpression may contrib-

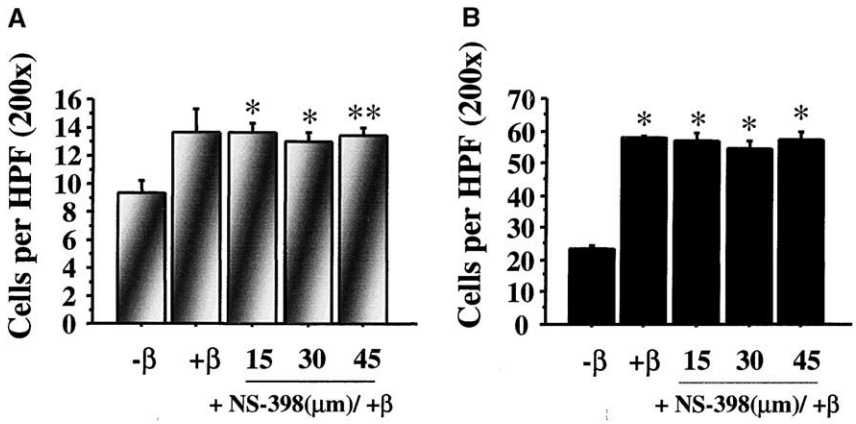


Fig. 5. Boyden chamber invasion assay analysis of YAMC and YAMC-Ras cells after TGF-β_1 and NS-398 treatment. YAMC and YAMC-Ras cells (1×10^4) were seeded onto type 1 collagen gel and treated with TGF-β_1 (5 ng/ml) and NS-398 (15 to 45 μm) for 24 hours. Membranes were preserved and cells were fixed and stained with methyl alcohol and crystal violet. **A**, Cell counts per high-power field for YAMC cells (*$P <$ 0.022; **$P < 0.04$). **B**, Cell counts per high-power field for YAMC-Ras cells (*$P < 0.0003$).

ute to the tumorigenic potential of dysplastic intestinal epithelial cells by enhancing adhesion to the extracellular matrix and by inhibition of apoptosis.[40] Expression of COX-2 also serves to increase the invasiveness and metastatic potential in human colon cancer ($CaCO_2$) cells,[41] which is in contrast to our present results. The cause of this different invasive response is unclear but may be attributed to major differences in the genetic makeup of these cell lines.

Activated Ras oncogene may have multiple important roles in tumorigenesis. One of these roles is the induction of COX-2 expression. We have previously demonstrated induction of COX-2 and increased PGE_2 production in Ha-Ras–transfected Rat-1 cells[20] and RIE cell lines.[14] Also, previous studies in non–small cell lung cancer cells demonstrated that Ras activation increased the expression of cytosolic phospholipase A, an enzyme that releases arachidonic acid from cellular phospholipid stores, thus allowing it to be available for the cyclooxygenase enzymes.[42] Together these observations suggest that increased prostanoid production may be an important phenotypic consequence of Ras activation. In this context it is interesting that activated Ras sensitizes cells to TGF-β–mediated COX-2 induction, as we have observed in small intestinal epithelial cells[14] and now in mouse colonocytes.

Transformation of cells to the invasive phenotype with metastatic potential is characterized by several alterations in gene expression and the cell and tissue plasticity. TGF-β has been demonstrated to be a potent modulator of epithelial to mesenchymal transition for keratinocytes and mammary cells.[43–46] In the present study we have demonstrated the increase in invasive potential of these cells in the setting of Ras activation, and this is enhanced after treatment with TGF-β_1. COX-2 enzyme inhibition with NS-398 failed to downregulate invasiveness in either cell line, suggesting that downstream effects of Ras activation and TGF-β signaling other than prostaglandin production contributes to the invasive phenotype.

CONCLUSION

Colorectal carcinogenesis often involves both activated Ras signaling and increased expression of TGF-β. Additionally, there is evidence that COX-2 plays an important role in early intestinal neoplasia and that TGF-β_1 is a potent inducer of COX-2 expression. In this study we have demonstrated that induction of COX-2 and eicosanoid release were augmented by activated Ras and TGF-β_1 in mouse colonocytes. Both Ras and TGF-β signaling contribute to invasiveness in a collaborative manner. COX-2 activity appears to not be involved in the invasive phenotype induced by Ras and TGF-β.

REFERENCES

1. Beauchamp RD, Peeler MO, DuBois RN. Recent advances in the management of colorectal polyps. Curr Opin Gastroenterol 1996;12:12–17.
2. Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell 1998;93:705–716.
3. DuBois RN, Shao J, Tsujii M, Sheng H, Beauchamp RD. GI delay in cells overexpressing prostaglandin endoperoxide synthase-2. Cancer Res 1996;56:733–737.
4. Kudo T, Narisawa T, Abo S. Antitumor activity of indomethacin on methylazoxymethanol-induced large bowel tumors in rats. Gann 1980;71:260–264.
5. Pollard M, Luckert PH. Effect of indomethacin on intestinal tumors induced in rats by the acetate derivative of dimethylnitrosamine. Science 1981;214:558–559.
6. Thun MJ, Namboodiri NM, Health CW Jr. Aspirin use and reduced risk of fatal colon cancer. N Engl J Med 1991; 325:1593–1596.
7. Giovannucci E, Egan KM, Hunter DG, Stampfer MJ, Colditz GA, Willet WC, Speizer FE. Aspirin and the risk of colorectal cancer in women. N Engl J Med 1995;333:609–614.
8. Smalley W, Ray WA, Daugherty J, Griffin MR. Use of nonsteroidal anti-inflammatory drugs and incidence of colorectal cancer: A population-based study. Arch Intern Med 1999;159:161–166.
9. Labayle D, Fischer D, Viehl P, Drouhin F, Pariente A, Bories C, Duhamel O, Trousset M, Attali P. Sulindac causes regression of rectal polyps in familial adenomatous polyposis. Gastroenterology 1991;101:635–639.
10. Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, Booker SV, Robinson CR, Offerhaus GJ. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. N Engl J Med 1993;328:1313–1316.
11. Nugent KP, Farmer KC, Spigelman AD, Williams CB, Phillips RK. Randomized controlled trial of the effect of sulindac on duodenal and rectal polyposis and cell proliferation in patients with familial adenomatous polyposis. Br J Surg 1993;80:1618–1619.
12. Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM, Evans JF, Taketo MM. Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase-2 (COX-2). Cell 1996;87: 803–809.
13. Sheng H, Shao J, Kirkland SC, Isakson P, Coffey RJ, Morrow J, Beauchamp RD, DuBois RN. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. J Clin Invest 1997;99:2254–2259.
14. Sheng H, Shao J, Dixon DA, Williams CS, Prescott SM, DuBois RN, Beauchamp RN. Transforming growth factor-beta 1 enhances Ha-Ras-induced expression of cyclooxygenase-2 in intestinal epithelial cells via stabilization of mRNA. J Biol Chem 2000;275:6628–6635.
15. Matsura H, Sakaue M, Subbaramaiah K, Kamitani H, Eling TE, Dannenberg AJ, Tanabe T, Inoue H, Arata J, Jettem AM. Regulation of cyclooxygenase-2 by interferon gamma and transforming growth factor alpha in normal human epidermal keratinocytes and squamous carcinoma cells. Role of mitogen-activated protein kinases. J Biol Chem 1999;274: 29138–29148.

16. Jones MK, Sasaki E, Halter F, Pai R, Nakamura T, Arakawa T, Kuroki T, Tamawski AS. HGF triggers activation of COX-2 gene in rat gastric epithelial cells: Action mediated through the ERK2 signaling pathway. FASEB 1999;13:2186–2194.
17. Xie W, Herschman HR. Transcriptional regulation of prostaglandin synthase 2 gene expression by platelet-derived growth factor and serum. J Biol Chem 1996;271:31742–31748.
18. Xie W, Herschman HR. v-src induces prostaglandin synthase 2 gene expression by activation of the c-Jun-N-terminal kinase and the c-Jun transcription factor. J Biol Chem 1995;270:27622–27628.
19. Perkins DJ, Kniss DA. Rapid and transient induction of cyclo-oxygenase 2 by epidermal growth factor in human amnion-derived WISH cells. Biochem J 1997;321:677–681.
20. Sheng H, Williams CS, Shao J, Liang P, DuBois RN, Beauchamp RD. Induction of cyclooxygenase-2 by activated Ha-ras oncogene in Rat-1 fibroblasts and the role of mitogen-activated protein kinase pathway. J Biol Chem 1998;273: 22120–22127.
21. Subbaramiah K, Chung WJ, Dannenberg AJ. Ceramide regulates the transcription of cyclooxygenase-2. Evidence for involvement of extracellular signal-regulated kinase/c-Jun N-terminal kinase and p38 mitogen-activated protein kinase pathways. J Biol Chem 1998;273:32943–32949.
22. Massague J. TGF-β signal transduction. Annu Rev Biochem 1998;67:753–791.
23. Engel ME, Datta PK, Moses HL. RhoB is stabilized by transforming growth factor β and antagonizes transcriptional activation. J Biol Chem 1998;273:9921–9926.
24. Hata A, Shi Y, Massague J. TGF-β signaling and cancer: Structural and functional consequences of mutations in Smads. Mol Med Today 1998;4:257–262.
25. Friedman E, Gold LI, Klimstra D, Zeng ZS, Winawer S, Cohen A. High levels of transforming growth factor beta 1 correlate with disease progression in human colon cancer. Cancer Epidemiol Biomarkers Prev 1995;4:549–554.
26. Beauchamp RD, Lyons RM, Yang EY, Coffey RJ Jr, Moses HL. Expression of and response to growth regulatory peptides by two human pancreatic carcinoma cell lines. Pancreas 1990;5:369–380.
27. Arteaga CL, Carty-Dugger T, Moses HL, Hurd SD, Pietenpol JA. Transforming growth factor beta 1 can induce estrogen-independent tumorigenicity of human breast cancer cells in athymic mice. Cell Growth Differ 1993;4:193–201.
28. Sun L, Wu G, Willson JK, Zborowska E, Yang J, Rajkarunanayake I, Wang J, Gentry LE, Wang WF, Brattain MG. Expression of transforming growth factor beta type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells. J Biol Chem 1994;269:26449–26455.
29. Hoosein NM, McKnight MK, Levine AE, Mulder KM, Childress KE, Brattain DE, Brattain MG. Differential sensitivity of subclasses of human colon carcinoma cell lines to the growth inhibitory effects of transforming growth factor-beta 1. Exp Cell Res 1989;181:442–453.
30. Forrester K, Almoguera C, Han K, Grizzle WE, Perucho M. Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. Nature 1987;327:298–303.
31. Bos JL, Fearon ER, Hamilton SR, Verlaan-de Vries M, van Boom JH, van der Eb AJ, Vogelstein B. Prevalence of ras gene mutations in human colorectal cancers. Nature 1987; 327:293–297.
32. Filmus J, Zhao J, Buick RN. Overexpression of H-ras oncogene induces resistance to the growth-inhibitory action of transforming growth factor beta-1 (TGF-β1) and alters the number and type of TGF-beta-1 receptors in rat intestinal epithelial cell clones. Oncogene 1992;7:521–526.
33. Winesett MP, Ramsey GW, Barnard JA. Type II TGF (beta) receptor expression in intestinal cell lines and in the intestinal tract. Carcinogenesis 1996;17:989–995.
34. Whitehead RH, Van Eeden PE, Noble MD, Ataliotis R, Jat PS. Establishment of conditionally immortalized cell lines from both colon and small intestine of adult H-2K^b-tsA58 transgenic mice. Proc Natl Acad Sci USA 1993;90:587–591.
35. Saha D, Datta PK, Sheng H, Morrow JD, Wada M, Moses HI, Beauchamp RD. Synergistic induction of cyclooxygenase-2 by transforming growth factor-beta 1 and epidermal growth factor inhibits apoptosis in epithelial cells. Neoplasia 1999;1:508–517.
36. Gilbert RS, Reddy ST, Kujubu DA, Xie W, Luner S, Herschman HR. Transforming growth factor beta 1 augments mitogen-induced prostaglandin synthesis and expression of the TIS10/prostaglandin synthase 2 gene both in Swiss 3T3 cells and in murine embryo fibroblasts. J Cell Physiol 1994;159:67–75.
37. Gilbert RS, Reddy ST, Targan S, Herschamn HR. TGF-beta 1 augments expression of the TIS10/prostaglandin synthase-2 gene in intestinal epithelial cells. Cell Mol Biol Res 1994;40:653–660.
38. Sheng H, Shao J, Hooton EB, Tsujii M, DuBois RN, Beauchamp RD. Cyclooxygenase-2 induction and transforming growth factor beta growth inhibition in rat intestinal epithelial cells. Cell Growth Differ 1997;8:463–470.
39. Sheng H, Shao J, O'Mahony CA, Lamps L, Albo D, Isakson PC, Berger DH, DuBois RN, Beauchamp RD. Transformation of intestinal epithelial cells by chronic TGF-β_1 treatment results in downregulation of the type II TGF-β receptor and induction of cyclooxygenase-2. Oncogene 1999; 18:855–867.
40. Tsujii M, Dubois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell 1995;83:493–501.
41. Tsujii M, Kawano S, DuBois RN. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc Natl Acad Sci USA 1997;94:3336–3340.
42. Heasley LE, Thaler S, Nick M, Price B, Skorecki K, Nemenoff RA. Induction of cytosolic phospholipase A2 by oncogenic Ras in human non-small cell lung cancer. J Biol Chem 1997;272:14501–14504.
43. Caulin C, Scholl FG, Frontelo P, Gamallo C, Quintanilla M. Chronic exposure of cultured transformed mouse epidermal cells to transforming growth factor-beta 1 induces an epithelial-mesenchymal transdifferentiation and a spindle tumoral phenotype. Cell Growth Differ 1995;6:1027–1035.
44. Zambruno G, Marchiso PC, Marconi A, Vascieri C, Melchiori A, Giannetti A, De Luca M. Transforming growth factor-beta 1 modulates beta 1 and beta 5 integrin receptors and induces the de novo expression of the alpha v beta 6 heterodimer in normal human keratinocytes: Implications for wound healing. J Cell Biol 1995;129:853–865.
45. Oft M, Peli J, Rudaz C, Schwarz H, Beug H, Reichmann E. TGF-beta 1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. Genes Dev 1996;10:2462–2477.
46. Oft M, Heider KH, Beug H. TGF-beta signaling is necessary for carcinoma cell invasiveness and metastasis. Curr Biol 1998;8:1243–1252.

Postprandial Absorptive Augmentation of Water and Electrolytes in the Colon Requires Intraluminal Glucose

Michael L. Kendrick, M.D., Nicholas J. Zyromski, M.D., Toshiyuki Tanaka, M.D., Judith A. Duenes, B.A., Karen Libsch, M.D., Michael G. Sarr, M.D.

Postprandial absorptive augmentation of water and electrolytes occurs in the small intestine and colon. The role of intraluminal nutrients in this response is poorly understood. Our aim was to determine whether postprandial absorptive augmentation of water and electrolytes in the colon requires the presence of intraluminal glucose. Four adult dogs underwent enteric isolation of a 50 cm segment of proximal colon. An ileal-like electrolyte solution (Na^+, 130 mEq/L; K^+, 10 mEq/L; Cl^-, 115 mEq/L; and HCO_3^-, 25 mEq/L), alone or containing glucose (10 mmol/L), was infused at 4 ml/min into the colonic segment. Experiments were performed during fasting and postprandially after a 400 Kcal mixed-nutrient meal. Effluent was collected in 60-minute intervals after steady state was achieved. Net absorptive flux of water was increased in the presence of intraluminal glucose during the fasted state (11 ± 0.8 vs 7.4 ± 0.9 μl/min/cm, $P < 0.01$). The net absorptive flux of water and electrolytes increased postprandially only in the presence of intraluminal glucose ($P < 0.05$). Our finding that glucose augments both baseline and postprandial absorption of water and electrolytes in the proximal colon suggests that luminal factors have a role in postprandial absorptive augmentation. Whether this is specific to glucose or occurs with other nutrients remains to be determined. (J GASTROINTEST SURG 2002;6:310-315.)

KEY WORDS: Colon, electrolyte, absorption, postprandial, glucose

In the postprandial state, absorption of water and electrolytes is augmented in the small and large intestine.[1–5] This postprandial absorptive augmentation has been well described in the small intestine. The specific segment of small intestine (jejunum vs. ileum), luminal constituents, extrinsic neural innervation, and caloric content of the meal all contribute to the magnitude of this response.[1,2,6,7] The response is predominantly initiated by luminal nutrients in the jejunum,[1,3] and the precise mechanisms of water and electrolyte absorption are largely dependent on Na^+/glucose and Na^+/H^+ transporters.[8–11]

In the colon, however, the phenomenon of postprandial absorptive augmentation is less clearly defined. With the use of short, 20 cm segments of proximal or distal colon, it has been shown that postprandial absorptive augmentation does exist in the colon.[4,5] Pilot attempts to reproduce these findings in our laboratory failed to demonstrate a postprandial absorptive augmentation in the proximal colon (unpublished data). However, one major difference in our study design was the omission of glucose or other nutrients from the test solution infused into the colon. Based on this observation, we hypothesized that postprandial augmentation of absorption of water and electrolytes does not occur in the absence of intraluminal glucose. The following experiments were designed to determine whether intraluminal glucose is required for the postprandial absorptive augmentation of water and electrolytes in the proximal canine colon.

METHODS

Experimental Design

Four adult mongrel dogs underwent complete enteric isolation of the proximal 50 cm of colon. Colonic

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2002 (poster presentation) and published as an abstract in *Gastroenterology* 120:A342, 2001.

From the Gastroenterology Research Unit, Department of Surgery (M.L.K., N.J.Z., T.T., J.A.D., K.L., M.G.S.), Mayo Clinic and Mayo Foundation, Rochester, Minnesota.

Reprint requests: Michael G. Sarr, M.D., Professor of Surgery, Gastroenterology Research Unit (AL 2-435), Mayo Clinic 200 First Street SW, Rochester, MN 55905.

Published by Elsevier Science Inc.

1091-255X/02/$—see front matter
PII: S1091-255X(01)00069-5

absorption of water and electrolytes was determined during fasting and postprandial states using an ileal-like electrolyte infusate with or without glucose.

Canine Preparation

Healthy female dogs, weighing 18 to 22 kg, were used according to the standards set forth by the Institutional Animal Care and Use Committee of Mayo Foundation in accordance with the guidelines of the National Institutes of Health and the United States Public Health Service policy on the humane care and use of laboratory animals. Dogs were anesthetized by means of intravenous sodium methohexital (12.5 mg/kg) and maintained with endotracheal isoflurane. All dogs underwent a two-stage operation similar to our previous preparation to study colonic absorption.[12] Stage 1 consisted of a short, lower midline celiotomy with ligation of the mesenteric colonic vascular arcade just proximal to the inferior mesenteric vein. This maneuver promotes diversion of venous drainage of the more distal colon through the inferior mesenteric and rectal venous pathways rather than proximally via the middle colic vein, thereby preventing venous congestion after proximal colonic isolation.[12] Dogs were allowed to recover for 1 week before stage 2, in which the colon was transected both at the ileocecal junction after excision of the cecum (equivalent to the human appendix) and just proximal to the site of the previous mesenteric arcade ligation, thereby enterically isolating a 50 cm segment of proximal colon. Enteric continuity was restored via an ileo-descending colostomy. The enterically isolated colonic segment was then constructed as a modified Thiry-Vella loop. An infusion catheter (outside diameter = 2 mm) was inserted into the proximal end of the segment and sutured in place, embedded into a metal cannula, and exteriorized through the right lateral abdominal wall. The distal end of the colonic segment was exteriorized through the left lateral abdominal wall as a standard colostomy. For the first 2 to 3 days, the dogs were given scheduled doses of intramuscular butorphanol for pain and maintained on parenteral fluids and electrolytes before ad libitum feeding was allowed. After a 2-week recovery, absorptive experiments were begun.

Conduct of Experiments

All absorptive experiments were conducted in conscious dogs resting comfortably in a Pavlov sling after an overnight fast. Two separate infusate solutions were used: (1) a basal, ileal-like, isosmolar solution, containing Na^+, 130 mEq/L; K^+, 10 mEq/L; Cl^-, 115 mEq/L; HCO_3^-, 25 mEq/L; and polyethylene glycol (PEG), 5 g/L, labeled with ^{14}C-PEG, 5 μCi/L; and (2) the same basal solution but also containing 10 mmol/L glucose. The prewarmed (38° C) solutions were infused continuously at 4 ml/min via the exteriorized catheter into the proximal end of the colonic segment (Fig. 1). All effluent was collected from the distal stoma and analyzed at specified intervals. For the first hour, all effluent was collected in 15-minute intervals to confirm steady-state conditions using percentage recovery of the nonabsorbable marker ^{14}C-PEG (Table 1). Steady-state conditions were defined by a recovery of more than 90% of the total amount of marker infused per interval; thereafter, all subsequent samples were used for absorptive calculations. After establishment of a steady state, effluent was collected for an additional 1 hour during the fasted state. Dogs were then fed a 400 Kcal mixed-nutrient meal (Alpo; Friskies Company Inc., Glendale, California), which all dogs ate regularly within approximately 2 minutes. Effluent was then collected in 60-minute intervals for 4 hours postprandially. Absorptive experiments were repeated on four separate days for each test solution (8 experiments per dog).

Analytic Methods

Concentrations of sodium and potassium were determined using flame photometry (Beckman Kline Flame; Beckman Instruments, Fullerton, California) and chloride using a chloridometer (Corning 920 M Chloridometer; Corning Scientific Instruments, Medfield, Massachusetts). Concentrations of the nonabsorbable marker ^{14}C-PEG were determined by liquid scintillation (Beckman LS 7800; Beckman Instruments). Net absorptive fluxes of water (μl/min/cm) and electrolytes (μEq/min/cm) were calculated using standard formulas (see Table 1). The mean individual values of net absorption for each dog over the four experiments for each test solution were calculated. A final mean was then calculated across all dogs for each test solution.

Statistical Analysis

Comparisons between test solutions were performed during both fasted and fed conditions using a paired Student's *t* test. Data are presented as mean ± SEM.

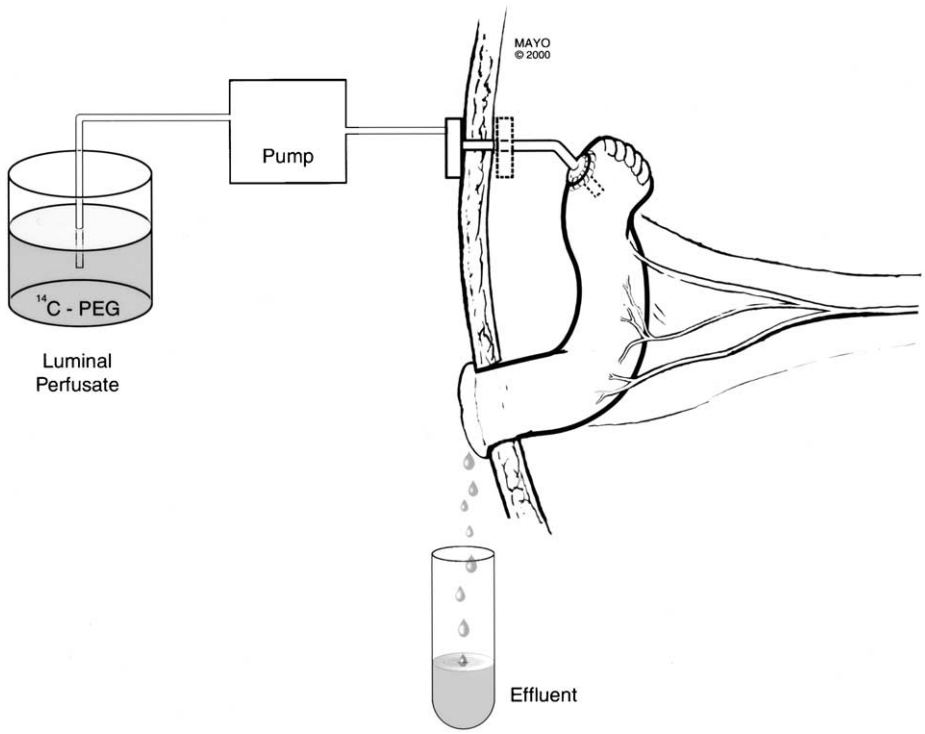


Fig. 1. Technique of infusion and recovery of simple electrolyte solution into an enterically isolated colonic segment.

RESULTS

General Health of Animals

All dogs remained healthy with formed stools and similar subjective health for the duration of the study. No obvious differences were noted between dogs in terms of appearance of the colonic stoma or in baseline output between experiments.

Fasting Absorption

Net absorptive fluxes of water during fasting, expressed as μl/min/cm, are shown in Fig. 2. In the fasted state, net absorptive flux of water was greater ($P < 0.01$) in the presence of intraluminal glucose (see Fig. 2). Similarly, the net absorptive flux of Na^+ and Cl^- absorption was greater when glucose was present in the infusate (Figs. 3 and 4). Net absorptive flux of K^+ was negative (representing a net secretion into the lumen) and did not differ between the two test solutions (Fig. 5).

Postprandial Absorption

Net absorption flux of water postprandially, expressed as μl/min/cm, is shown in Fig. 2. Postprandially, the net absorptive flux of water did not differ from the fasted state when the segment was infused with basal solution that did not contain glucose. In contrast, in the presence of intraluminal glucose, postprandial absorption of water increased in the first hour and continued to increase each hour, up to 4 hours after feeding ($P < 0.01$). In addition, the presence of intraluminal glucose increased the net absorptive flux of water during each postprandial time period compared to basal solution without glucose ($P < 0.001$).

Net absorptive fluxes of sodium and chloride were unchanged postprandially in the absence of glucose

Table 1. Recovery and net absorptive flux calculations for water and electrolytes

Recovery =	[^{14}C-PEG] effluent × volume effluent/[^{14}C-PEG] infusate × volume infusate
Flux $_{H_{20}}$ =	PR × 1 − [^{14}C-PEG] infusate/[^{14}C-PEG] effluent
Flux ion =	PR × [Ion] infusate − [Ion] effluent × [^{14}C-PEG] infusate/[^{14}C-PEG] effluent

PEG = polyethylene glycol; PR = perfusion rate.

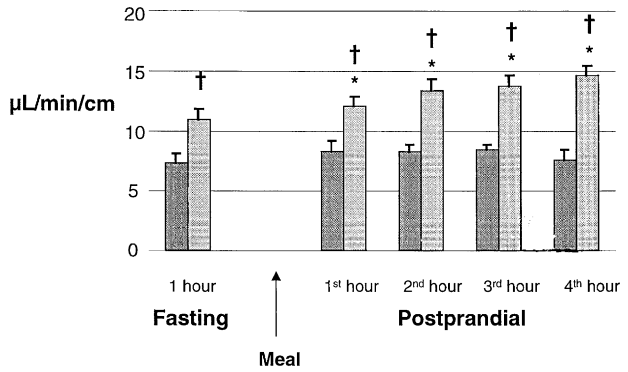


Fig. 2. Net absorptive flux of H_2O during fasting and postprandial states using colonic infusate with and without glucose. Dark gray = basal; light gray = basal plus glucose; n = 4; *$P < 0.01$ vs. fasting within each group; †$P < 0.01$ vs. basal solution (mean ± SEM).

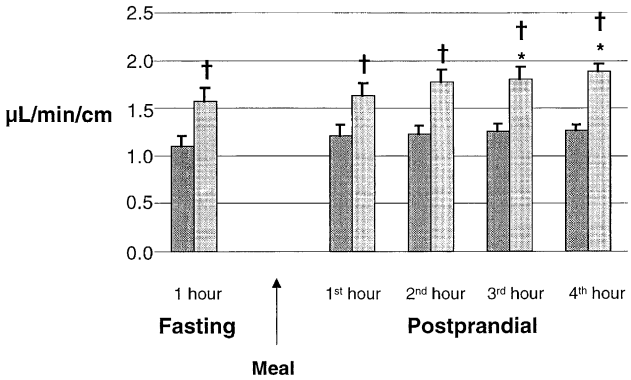


Fig. 4. Net absorptive flux of Cl^- during fasting and postprandial states using colonic infusate with and without glucose. Dark gray = basal; light gray = basal plus glucose; n = 4; *$P < 0.01$ vs. fasting within each group; †$P < 0.01$ vs. basal solution (mean ± SEM).

but were increased in the presence of intraluminal glucose, similar to the change in water flux (see Figs. 3 and 4). Net flux of potassium did not change postprandially with either solution (Fig. 5).

DISCUSSION

This study demonstrates that postprandial absorptive augmentation in the canine proximal colon does not occur with the use of ileal-like electrolyte solutions devoid of nutrients; however, the presence of intraluminal glucose provides a permissive mechanism to allow the postprandial absorptive augmentation of water and electrolytes to occur. In addition, we have shown that intraluminal glucose enhances baseline colonic absorption of water and electrolytes during both fasted and postprandial states.

After a meal, there is an increased absorption of water and electrolytes in the small intestine known as postprandial absorptive augmentation. This physiologic response has been well characterized. The magnitude of the response is greater in the ileum than in the jejunum[2] and is greater with increasing caloric content of the meal.[1] The signal for this response appears to originate predominantly from the jejunum by intraluminal nutrients[1,3] and is altered but not abolished by loss of extrinsic neural innervation.[6,7] In addition, cholinergic and opioid pathways appear to contribute to the response.[13–15] Sodium transporters have been implicated in the mechanisms mediating

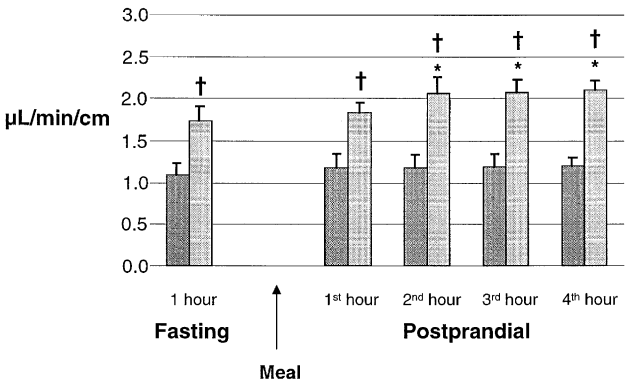


Fig. 3. Net absorptive flux of Na^+ during fasting and postprandial states using colonic infusate with and without glucose. Dark gray = basal; light gray = basal plus glucose; n = 4; *$P < 0.01$ vs. fasting within each group; †$P < 0.01$ vs. basal solution (mean ± SEM).

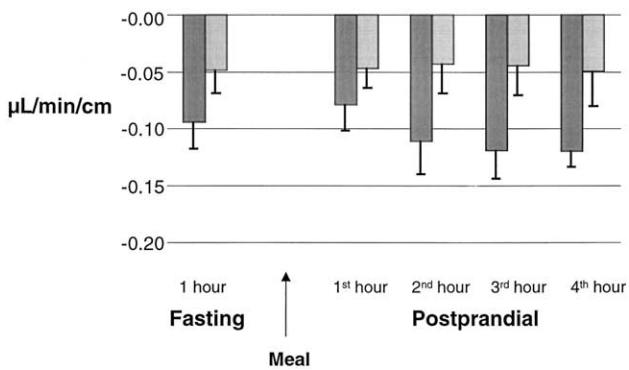


Fig. 5. Net absorptive flux of K^+ during fasting and postprandial states using colonic infusate with and without glucose. Dark gray = basal; light gray = basal plus glucose; n = 4; * $P <$ 0.01 vs. fasting within each group; † $P < 0.01$ vs. glucose solution (mean ± SEM).

the augmented absorption of water and electrolytes. In the jejunum, the Na^+/glucose transporter appears to mediate this response.[10,11] In the ileum, however, the Na^+/H^+ transporter appears responsible for the absorptive augmentation, as blocking the Na^+/H^+ transporter abolishes the postprandial absorptive augmentation.[8,9]

A postprandial absorptive augmentation has also been described in the colon[4,5]; however, the mechanisms mediating this response are poorly characterized. It is unknown whether this response is sensitive to specific anatomic regions of the colon, caloric content of the oral meal, extrinsic innervation, or intraluminal nutrients, or whether certain transport processes mediate this response. Using an isolated 20 cm distal colonic segment, Ashton et al.[4] demonstrated a postprandial absorptive augmentation in the distal canine colon. This response has also been demonstrated in the proximal canine colon[16] and proximal colon of the pig.[5]

In our laboratory, pilot experiments were unable to validate this response in an isolated 50 cm segment of proximal colon (unpublished data). We surmised that this discrepancy was based on differences in experimental design. Previous studies have used in vivo models of short, 20 to 25 cm colonic test segments. Our model used a 50 cm segment, perhaps reflecting a more overall colonic effect. More important, previous studies used test solutions containing nutrients such as glucose, other carbohydrates, or normal ileal effluent. Conversely, our previous experiment used an ileal-like electrolyte solution devoid of nutrients. Thus we hypothesized that glucose may afford a permissive role in the postprandial response. The aim of this study was to determine whether the presence of intraluminal glucose was necessary for the postprandial absorptive augmentation of water and electrolytes in the canine proximal colon.

We have shown that a postprandial absorptive augmentation of water and electrolytes does not occur with an ileal-like electrolyte solution devoid of nutrients. When glucose alone was added to the electrolyte solution, an increase in absorption of water and electrolytes was observed similar in character to previous reports of postprandial absorptive augmentation. These findings suggest that intraluminal glucose is necessary for the postprandial absorptive response.

The potential mechanisms by which intraluminal glucose may permit postprandial augmented absorption are intriguing. Unlike the small intestine, the colon has no specific receptors for absorption of sugars. Long et al.[17] demonstrated, more than 30 years ago, that glucose absorption in the human colon is insignificant or zero. Although Hines et al.[18] demonstrated a small amount of SGTL1 messenger RNA expression in rat colonocytes after small bowel resection, there are no data to suggest the presence of specific glucose receptors or absorption of glucose in the colon. Rather, unabsorbed carbohydrate is largely converted to volatile fatty acids by the anaerobic bacterial flora.[19–21]

Whether this effect is specific for glucose only, or whether other nutrients (carbohydrates, short-chain fatty acids, amino acids, etc.) are capable of this permissive effect in the postprandial absorptive augmentation, cannot be determined from this study. The products of carbohydrate fermentation in the colon, short-chain fatty acids, may be responsible for this response. N-butyrate, the predominant fuel source for the colonocyte, is one plausible explanation. Butyrate is important in the absorption of Na^+ via the electroneutral Na^+/H^+—Cl^-/HCO_3^- exchange, which is thought to predominate in the absorption of sodium from the proximal colon.[22,23] Short-chain fatty acids are thought to increase Na^+ absorption by recycling of H^+ within the cell as short-chain fatty acids enter the colonocyte. This H^+ is then used in the Na^+/H^+ exchange to transport Na^+ into the cell.[24] Although this seems plausible, bacterial decontamination of the colon reduced the formation of volatile fatty acids by 90%, yet did not affect postprandial water absorption.[5]

Peptide YY has been shown to play a role in colonic postprandial absorptive augmentation. Both intravenous and intraluminal peptide YY augment colonic absorption in vivo.[16,25] Immunoneutralization of circulating peptide YY with peptide YY antibody inhibits the postprandial absorptive augmentation, suggesting its potential role in this response. Further, cholecystokinin stimulates release of peptide YY, whereas MK329, a receptor antagonist, blunts the colonic postprandial absorptive augmentation.[26] These findings highlight the fact that humoral factors, in addition to luminal factors (glucose), are important in mediating the colonic postprandial absorptive augmentation.

We have also shown that intraluminal glucose enhances baseline absorption of water and electrolytes. The influence of intraluminal glucose in baseline water and electrolyte absorption in the colon is controversial. In vitro experiments have not consistently demonstrated an effect of glucose on baseline water and electrolyte absorption.[27–29] Few in vivo studies are available that specifically evaluate the effects of glucose or its absence in colonic absorption, and results of these studies are confounded by the use of other carbohydrates such as mannitol, which undergoes bacterial fermentation to glucose in the colon.[28,30] Our findings clearly demonstrate that intraluminal

glucose increases both the fasted and postprandial baseline absorption of water and electrolytes in an in vivo model. This finding is intriguing, and whether glucose or the products of its fermentation stimulate sodium transporters, promote secretion of peptide YY, or mediate some other absorptive mechanisms is unknown.

CONCLUSION

We have demonstrated that intraluminal glucose enhances both fasting and postprandial absorption of water and electrolytes, and has a permissive role in the postprandial absorptive augmentation of water and electrolytes in the proximal canine colon. Whether glucose, specifically, or the products of fermentation mediate this response remains to be determined.

We thank Deborah I. Frank for her secretarial assistance, and Ethicon, Inc., for the surgical staplers used in the preparation of the dog model.

REFERENCES

1. Anthone GJ, Zinner MJ, Yeo CJ. Small bowel origin and calorie dependence of a signal for meal-induced jejunal absorption. Ann Surg 1993;217:57–63.
2. Anthone GJ, Wang BH, Zinner MJ, Yeo CJ. Site-specific variations in basal and meal-stimulated intestinal absorption. J Surg Res 1992;52:454–458.
3. Bastidas JA, Orandle MS, Zinner MJ, Yeo CJ. Small-bowel origin of the signal for meal-induced jejunal absorption. Surgery 1990;108:376–383.
4. Ashton KA, Chang LK, Anthone GJ, Ortega AE, Simons AJ, Beart RW. Basal and meal-stimulated colonic absorption. Dis Colon Rectum 1996;39:865–870.
5. Theodorou V, Fioramonti J, Bueno L. Water absorption from the pig proximal colon: Relations with feeding and flow of digesta. Q J Exp Physiol 1989;74:521–529.
6. Sarr MG, Kelly KA, Phillips SF. Feeding augments canine jejunal absorption via a hormonal mechanism. Dig Dis Sci 1981;26:961–965.
7. Barry KM, Aloisi JD, Yeo CJ. Neural mechanisms in basal and meal-stimulated ileal absorption. J Surg Res 1995;58: 425–431.
8. Yeo CJ, Varry KM, Gontarek JD, Donowitz M. Na/H exchange mediates meal-stimulated ileal absorption. Surgery 1994;116:388–395.
9. Hines OJ, Bilchik AJ, McFadden DW, Rodgers PJ, Bautista N, Zinner MJ, Ashley SW. Na/H exchange mediates postprandial ileal water and electrolyte transport. Dig Dis Sci 1995;40:774–780.
10. Welton ML, Ashley SW, Barquist ES, Miller JL, Zinner MJ. Role of Na/glucose cotransport in meal-induced jejunal absorption. Surg Forum 1992;48:147–148.
11. Hines OJ, Bilchik AJ, Zinner MJ, Lane J, Hirmand M, Welton ML, Ashley SW. The Na/glucose cotransporter modulates jejunal postprandial absorption [abstr]. Gastroenterology 1993;104:A252.
12. Kendrick ML, Meile T, Zyromski NJ, Tanaka T, Sarr MG. Extrinsic neural innervation mediates absorption of water and electrolytes in canine proximal colon in vivo. J Surg Res 2001;97:76–80.
13. Yeo CJ, Bastidas AJ, Schmieg RE, Zinner MJ. Meal-stimulated absorption of water and electrolytes in canine jejunum. Am J Physiol 1990;259:402–409.
14. McFadden DW, Jaffe BM, Ferrara A, Zinner MJ. Jejunal absorptive response to a test meal and its modification by cholinergic and calcium channel blockage in the awake dog. Surg Forum 1984;35:174–176.
15. Bastidas AJ, Yeo CJ, Schmieg RE, Zinner MJ. Endogenous opiates in the mediation of early meal-induced jejunal absorption of water and electrolytes. Am J Surg 1989;157:27–32.
16. Liu CD, Aloia T, Adrian TE, Newton TR, Bilchik AJ, Zinner MJ, Ashley SW, McFadden DW. Peptide YY: A potential proabsorptive hormone for the treatment of malabsorptive disorders. Am Surg 1996;62:232–236.
17. Long CL, Geiger JW, Kinney JM. Absorption of glucose from the colon and rectum. Metabolism 1967;16:413–418.
18. Hines OJ, Bilchik AJ, Zinner MH, Skotzko MJ, Moser AJ, McFadden DW, Ashley SW. Adaptation of the Na^+/glucose cotransporter following intestinal resection. J Surg Res 1994;57:22–27.
19. Read NW. The relationships between colonic motility and transport. Scand J Gastroenterol 1984;93(Suppl):35–42.
20. Cummings JH. Short chain fatty acids in the human colon. Gut 1981;22:763–779.
21. Cummings JH. Colonic absorption: The importance of short chain fatty acids in man. Scand J Gastroenterol 1984; 93:89–99.
22. Roediger WE. Short chain fatty acids as metabolic regulators of ion absorption in the colon. Acta Vet Scand 1989;86: 116–125.
23. Sandle GI. Mechanisms of colonic electrolyte absorption and secretion. Dig Dis 1988;6:1–14.
24. Sandle GI. Salt and water absorption in the human colon: A modern appraisal. Gut 1998;43:294–299.
25. Liu CD, Newton TR, Zinner MJ, Ashley SW, McFadden DW. Intraluminal peptide YY induces colonic absorption in vivo. Dis Colon Rectum 1997;40:478–482.
26. Liu CD, Hines OJ, Newton TR, Adrian TE, Zinner MJ, Ashley SW, McFadden DW. Cholecystokinin mediation of colonic absorption via peptide YY: Foregut-hindgut axis. World J Surg 1996;20:221–227.
27. Argenzio, RA Southworth M, Lowe JE, Stevens CE. Interrelationship of Na, HCO_3 and volatile fatty acid transport by equine large intestine. Am J Physiol 1988;233:469–478.
28. Campos MS, Galindo MC, Garcia JA, Lisbona F, Lopez-Aliaga I. A comparative study of electrolyte and water transport in the rabbit ileum and colon in vitro and in vivo: Influence of D-glucose. Biomed Biochim Acta 1991;50:279–283.
29. Sullivan SK, Smith PL. Bicarbonate secretion by rabbit proximal colon. Am J Physiol 1986;251:436–445.
30. Fleming LL, Floch MH. Digestion and absorption of fiber carbohydrate in the colon. Am J Gastroenterol 1986;81: 507–511.

Perioperative Resting Pressure Predicts Long-Term Postoperative Function After Ileal Pouch–Anal Anastomosis

Amy L. Halverson, M.D., Tracy L. Hull, M.D., Feza Remzi, M.D., Jeffery P. Hammel, Tom Schroeder, M.D., Victor W. Fazio, M.D.

The purpose of this study was to determine whether perioperative manometry is useful in predicting long-term functional status after ileal pouch–anal anastomosis (IPAA). Prospectively collected perioperative anal manometry data from 1439 patients undergoing IPAA from 1986 to 2000 were compared to postoperative functional status at various time intervals from 6 months to 8 years after IPAA. A validated questionnaire was used to obtain information regarding restrictions of diet, work, social and sexual activity, urgency, fecal seepage or incontinence, energy level, satisfaction with surgery, and quality of life. The presence of seepage and the degree of incontinence were compared to the patient's perceived quality of life, health, energy level, and satisfaction with surgery. Low (<40 mm Hg) pre- and postoperative resting pressures were associated with increased seepage, pad use, and incontinence. Patients with low resting pressures also reported diminished quality of life, health, energy level, and satisfaction with surgery. There was a significant association ($P < 0.001$) between seepage and degree of incontinence and quality of health, quality of life, energy level, and level of satisfaction with surgery. Perioperative resting anal sphincter pressures greater than 40 mm Hg are associated with significantly better function and quality of life after ileal IPAA. Improved functional outcome is associated with better quality-of-life outcomes. Low preoperative resting pressures do not preclude successful outcome after IPAA. (J GASTROINTEST SURG 2002;6:316–321.)

KEY WORDS: Ileal pouch, manometry, function, incontinence, anastomosis

The ileal pouch procedure is an alternative to a permanent end ileostomy for patients who require a total proctocolectomy. The majority of patients experience good continence after this procedure. However, approximately 25% to 30% will experience some degree of fecal incontinence, and 10% to 62% will experience a small amount of anal seepage.[1–7] Pouch failure due to incontinence has been reported in 6% to 30% of patients whose pouches have been excised or are nonfunctional. Overall pouch failure due to incontinence occurs in less than 1% of all patients undergoing IPAA.[6,8]

Although several studies have evaluated perioperative anal sphincter tone, pouch compliance, sensation, and pudendal nerve motor function, the extent to which each of these factors contributes to postoperative incontinence has not been well established.[9–15] In contrast to preoperative incontinence, in which active rectal disease is a contributing factor, incontinence after IPAA is generally not associated with urgency and is more likely the result of poor sphincter function.

At our institution, candidates for restorative proctocolectomy with ileal pouch–anal anastomosis (IPAA) routinely undergo perioperative manometric assessment of sphincter function. Although there is no specific level below which patients are refused surgery, identifying low anal sphincter pressures allows one to counsel patients about possible increased risk of seepage or frank incontinence. Also, patients with low anal sphincter pressures are instructed on performing Kegel exercises to help strengthen their sphincter muscles before completing the pouch procedure.

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (oral presentation).

From the Departments of Colorectal Surgery (A.L.H., T.L.H., F.R., T.S., V.W.F.) and Biostatistics (J.P.H.), The Cleveland Clinic Foundation, Cleveland, Ohio.

Reprint requests: Tracy L. Hull, M.D., Department of Colorectal Surgery, 9500 Euclid Ave., Desk A-30, Cleveland, OH 44195. e-mail: hullt@ccf.org

Published by Elsevier Science Inc.

1091-255X/02/$—see front matter
PII: S1091-255X(01)00072-5

We hypothesized that low perioperative anal sphincter pressures are associated with poorer postoperative functional outcome and that functional outcome correlates with patient's perceived quality of life.

MATERIAL AND METHODS

Preoperative anal sphincter manometry was routinely performed on patients before creation of an IPAA. Most patients had a diverting loop ileostomy created at the time of their pouch surgery and had manometric testing repeated before closure of the loop ileostomy. Anal sphincter pressures at rest and at maximal squeeze were measured with the patient in the lateral decubitus position using a four-channel water perfusion catheter. Each catheter port was connected to a pressure transducer, and the signal was displayed on a chart recorder (Medtronics, Inc., Minneapolis, Minnesota). Pressures are measured at 1 cm intervals with the patient at rest and after asking the patient to squeeze the anal sphincter. Normal anal sphincter pressures are considered to be greater than or equal to 40 mm Hg at rest and 100 mm Hg at maximal squeeze.

A previously validated, self-administered questionnaire was used to obtain information regarding urgency, fecal seepage, fecal incontinence, pad use, activity restrictions, and quality-of-life factors in patients with a functioning pelvic pouch.[8] Urgency was defined as inability to defer bowel movement for more than 30 minutes. Urgency and incontinence were classified as occurring "never, rarely, sometimes, mostly, or always." Patients responded to questions about seepage during the day or night, pad use, and restriction of diet, work, social activities, or sexual activities with "yes" or "no." On a scale of 1 to 10, with 10 being the best, patients were asked to rate their current quality of life, quality of health, energy level, and satisfaction with the results of surgery. Patients completed the questionnaire at the initial postoperative visit and at subsequent follow-up visits. Patients who had their long-term follow-up elsewhere were periodically sent questionnaires by mail or were contacted by telephone. All information was recorded prospectively in a computer database.

Statistical Analysis

Logistic regression analysis was used to compare pre- and postoperative resting and squeeze anal sphincter pressures to the presence of incontinence, seepage, pad use, and activity restrictions. Linear regression was used to compare the manometric data to the postoperative quality-of-life scores. *t* Tests were used to assess the statistical significance of associations between the functional status and quality-of-life outcomes. The magnitude of decrease from preoperative resting pressure compared to postoperative resting pressure was compared to functional outcome using linear regression analysis. We also compared sex and diagnosis to functional outcome by the logistic and linear regression methods. Functional and quality-of-life outcome was evaluated at various time intervals: 6 months (±2 months), 1 year (±4 months), 1.5 to 2.5 years, 2.5 to 5 years, and 5 to 8 years. When patients were seen more than once during a particular time interval, the follow-up point closest to the center of the interval was used for analysis. *P* values were corrected for age and sex with $P < 0.05$ considered significant.

RESULTS

Our IPAA database contained anal sphincter manometric information and follow-up questionnaire data from 2084 patients who underwent IPAA between 1986 and 2000. Both prepouch and preileostomy closure manometric data and information from at least one follow-up questionnaire were available for 1439 of patients (69%). For many patients, questionnaire data were available from more than one follow-up time interval (Table 1). Some questionnaires were not filled out completely, which accounts for the variation in patient numbers between the different symptom categories (Tables 2 and 3). Ulcerative colitis was the diagnosis in 1134 patients. Indeterminate colitis and Crohn's disease were the diagnoses in 139 and 67 patients, respectively. Ninety-eight patients had familial adenomatous polyposis. One patient had colonic aganglionosis (Hirschsprung's disease). The median age was 38 years (range 10 to 77 years). Males comprised 55.5% of the patients.

Low (<40 mm Hg) pre- and postoperative resting pressures were associated with increased rates of incontinence, daytime and night-time seepage, and pad use (see Table 2). The maximal squeeze anal sphincter pressures did not consistently correlate with outcome (Table 3). Patients experiencing incontinence or seepage reported a lower average quality of life, health, energy level, and degree of satisfaction with surgery (Table 4).

There was a significant ($P < 0.001$) decrease in median resting anal sphincter pressure from 77 mm Hg (range 13 to 175 mm Hg) preoperatively to 52 mm Hg (range 10 to 145 mm Hg) measured before closure of the ileostomy. There was a similar de-

Table 1. Number of patients at each follow-up time interval

	6 mo	1 yr	1.5–2.5 yr	2.5–5 yr	5–8 yr
N	621	699	801	963	710

crease in median squeeze pressure from 183 mm Hg (range 25 to 447 mm Hg) perioperatively to 154 mm Hg (range 24 to 440 mm Hg) measured before loop ileostomy closure ($P < 0.001$). When evaluating pre-pouch manometry with manometry done after pouch surgery, but before ileostomy closure, the sphincter pressures measured before the initial pouch surgery had a similar correlation with postoperative function and quality of life as did anal sphincter pressures measured before ileostomy closure.

A greater magnitude of the drop between pre- and postoperative (measured before ileostomy closure) resting pressures correlated with increased daytime seepage and incontinence at 6 months, 1 year, and the 1.5- to 2.5-year intervals. There was a significant association between the percentage drop in resting anal sphincter pressure and night-time seepage at all follow-up intervals except the 2.5- to 5-year interval. Patients with higher preoperative resting pressures were more likely to have a greater drop in pressure postoperatively; $r = 0.69$.

The perioperative resting pressures were not consistently predictive of diet restriction, social activity, work activity, or sexual activity. There was no significant difference when comparing sex or diagnosis of ulcerative colitis to familial adenomatous polyposis.

DISCUSSION

Our results show that perioperative manometry is useful for predicting both postoperative function and quality of life. These results support earlier studies in which low preoperative resting sphincter tone was demonstrated to correlate with postoperative continence.[10–15] Prior studies from this institution and elsewhere have demonstrated that worse functional outcome corresponds to a lower quality of life.[8,16] Other studies have been unsuccessful in demonstrating a correlation between perioperative resting pressures and outcome or between functional outcome and patients' perceived quality of life.[17–19] Our study has the advantages of having a large number of patients and of having patient follow-up at multiple time points using an ileal pouch–specific, validated questionnaire to assess quality of life and functional outcome.

Assessment of preoperative resting pressure allows the surgeon to better counsel patients regarding expected postoperative results, thus providing the patient with realistic expectations after pelvic pouch surgery. Low resting sphincter pressures may uncover intrinsic sphincter deficiencies previously clinically silent or not noticed because of other symptoms of the primary disease. Incontinence may become clinically significant after IPAA because of changes in other factors important in maintaining continence such as sensation, compliance, and pudendal nerve motor function. All of these factors are not routinely measured postoperatively in our patients after IPAA.

In a previous study from this institution, Church et al.[20] observed that the higher the preoperative resting anal sphincter pressure, the greater the drop

Table 2. Resting anal sphincter pressure vs. functional status

Outcome	Sphincter pressure (mm Hg)	6 mo	1 yr	1.5–2.5 yr	2.5–5 yr	5–8 yr
Incontinence	>40	27/121 (22.3%)	31/102 (30.4%)	45/138 (32.6%)	64/167 (38.3%)	52/135 (38.5%)
	<40	44/362 (12.2%)	72/305 (23.6%)	104/415 (25.1%)	132/510 (25.9%)	110/38.7 (28.4%)
	P value	0.001	0.003	0.001	<0.001	<0.001
Day seepage	>40	39/92 (37%)	29/88 (33%)	42/118 (35.6%)	41/153 (26.8%)	36/119 (30.3%)
	<40	36/272 (13.2%)	44/271 (16.2%)	53/360 (14.7%)	73/474 (15.2%)	56/37 (15%)
	P value	<0.001	0.001	<0.001	<0.001	<0.001
Night seepage	>40	48/93 (51.6%)	41/88 (46.6%)	58/118 (49.2%)	66/152 (43.4%)	59/121 (48.8%)
	<40	81/275 (29.5%)	79/277 (28.5%)	107/360 (29.7%)	165/478 (34.5%)	123/376 (32.7%)
	P value	<0.001	0.001	0.001	0.002	<0.001

Number with outcome/total number.

Table 3. Maximal-squeeze anal sphincter pressure vs. functional status

Outcome	Sphincter pressure (mm Hg)	6 mo	1 yr	1.5–2.5 yr	2.5–5 yr	5–8 yr
Incontinence	>100	24/235 (10.2%)	42/203 (28%)	55/266 (20.7%)	80/323 (24.8%)	73/264 (27.7%)
	<100	54/290 (18.6%)	77/275 (28%)	11/368 (30.2%)	145/480 (30.2%)	107/338 (31.7%)
	P value	0.22	0.03	0.06	0.001	0.003
Day seepage	>100	28/169 (16.6%)	28/170 (16.5%)	35/226 (15.5%)	46/294 (15.6%)	36/258 (14%)
	<100	59/228 (25.9%)	65/247 (26.3%)	70/327 (21.4%)	96/451 (21.3%)	72/324 (22.2%)
	P value	0.091	0.02	0.025	0.13	0.016
Night seepage	>100	53/173 (30.6%)	49/169 (29%)	70/224 (31.3%)	96/297 (32.3%)	86/258 (31%)
	<100	98/231 (42.4%)	91/252 (36.1%)	122/331 (36.9%)	169/449 (37.6%)	121/327 (37%)
	P value	0.062	0.21	0.28	0.25	0.01

Number with outcome/total number.

in the resting pressure after IPAA and that a greater drop in pressure correlated with worse functional outcome. Although we continue to observe this relationship, the pre- and postoperative resting pressures remain more consistent predictors of outcome than when compared to the change in pressure.

Although patients with a lower resting pressure are more prone to problems with seepage or incontinence, less than half of this subgroup will report either of these symptoms. Although there was a significant difference in quality-of-life scores between patients with low resting pressures (< 40 mm Hg) and those with normal resting pressure (> 40 mm Hg), the mean scores for patients with resting anal sphincter pressures less than 40 mm Hg were still greater than 8 on a scale from 1 to 10 at each follow-up interval. Thus, despite having lower resting pressures and quality-of-life scores, these patients do not view their quality of life negatively.

One weakness of this report is that there was no preoperative assessment of the patients' function and quality of life. Pemberton et al.[4] previously reported that patients with higher stool frequency preoperatively are more prone to postoperative incontinence. Other studies have reported improvement in anal sphincter pressures after loop ileostomy closure.[5,17] Anal sphincter manometric data were not routinely obtained after closure of the loop ileostomy. These data would have been useful to compare long-term changes in sphincter pressures with functional outcome.

Table 4. Mean quality-of-life outcomes vs. functional status

Functional status	Mean score for quality of: Life	Health	Energy	Satisfaction with surgery
Incontinence				
"Sometimes, mostly, always"	7.96	7.87	7.28	8.18
"Never, rarely"	8.96	8.71	8.13	9.28
Daytime seepage				
Yes	7.78	7.63	7.04	7.90
No	8.85	8.65	8.07	9.21
Night-time seepage				
Yes	8.17	8.08	7.41	8.49
No	8.93	8.68	8.15	9.24

P value 0.001 for each comparison of means by a *t* test. Values are computed on the basis of 1.5- to 2.5-year outcomes (available n = 801).

CONCLUSION

Perioperative resting anal sphincter pressures greater than 40 mm Hg are associated with significantly better function and quality of life after IPAA. Improved functional outcome is associated with better quality-of-life outcomes. Low preoperative resting pressures do not preclude successful outcome after IPAA.

We thank Jane Bast, R.N., Miriam Preen, R.N., and Elena Manilich for their time and dedication in collecting data and maintaining our ileal pouch database.

REFERENCES

1. Fazio VW, Ziv Y, Church JM, Oakley JR, Lavery IC, Milsom JW, Schroeder TK. Ileal pouch–anal anastomoses complications and function in 1005 patients. Ann Surg 1995;222:120–127.
2. McIntyre PB, Pemberton JH, Wolff BG, Dozois RR, Beart RW Jr. Indeterminate colitis. Long-term outcome in patients after ileal pouch–anal anastomosis. Dis Colon Rectum 1995;38:51–54.
3. Seidel SA, Peach SE, Newman M, Sharp KW. Ileoanal pouch procedures: Clinical outcomes and quality-of-life assessment. Am Surg 1999;65:40–46.
4. Pemberton JH, Kelly KA, Beart RW Jr, Dozois RR, Wolff BG, Ilstrup DM. Ileal pouch–anal anastomosis for chronic ulcerative colitis. Long-term results. Ann Surg 1987;206:504–513.
5. Stelzner M, Fonkalsrud EW. Assessment of anorectal function after mucosal proctectomy and endorectal ileal pull-through for ulcerative colitis. Surgery 1990;107:201–208.
6. Marcello PW, Roberts PL, Schoetz DJ, Coller JA, Murray JJ, Veidenheimer MC. Long-term results of the ileoanal pouch procedure. Arch Surg 1993;128:500–504.
7. Meagher AP, Farouk R, Dozois RR, Kelly KA, Pemberton JH. J ileal pouch–anal anastomosis for chronic ulcerative colitis: Complications and long-term outcome in 1310 patients. B J Surg 1998;85:800–803.
8. Fazio VW, O'Riordain MG, Lavery IC, Church JM, Lau P, Strong SA, Hull T. Long-term functional outcome and quality of life after stapled restorative proctocolectomy. Ann Surg 1999;230:575–586.
9. Goes R, Beart RW Jr. Physiology of ileal pouch–anal anastomosis. Current concepts. Dis Colon Rectum 1995;38: 996–1005.
10. Nasmyth DG, Johnston D, Godwin PG, Dixon MF, Smith A, Williams NS. Factors influencing bowel function after ileal pouch–anal anastomosis. B J Surg 1986;73:469–473.
11. Nicholls RJ, Belliveau P, Neill M, Wilks M, Tabaqchali S. Restorative proctocolectomy with ileal reservoir: A pathophysiological assessment. Gut 1981;22:462–468.
12. O'Connell PR, Stryker SJ, Metcalf AM, Pemberton JH, Kelly KA. Anal canal pressure and motility after ileoanal anastomosis. Surg Gynecol Obstet 1988;166:47–54.
13. Heppell J, Taylor BM, Beart RW Jr, Dozois RR, Kelly KA. Predicting outcome after endorectal ileoanal anastomosis. Can J Surg 1983;26:132–134.
14. Ferrara A, Pemberton JH, Grotz RL, Hanson RB. Motor determinants of incontinence after ileal pouch–anal anastomosis. B J Surg 1994;81:285–288.
15. Scott NA, Pemberton JH, Barkel DC, Wolff BG. Anal and ileal pouch manometric measurements before ileostomy closure are related to functional outcome after ileal pouch–anal anastomosis. B J Surg 1989;76:613–616.
16. Steens J, Meijerink WJ, Masclee AA, van Hogezand RA, Griffioen G, Post WJ, Bemelman WA. Limited influence of pouch function on quality of life after ileal pouch–anal anastomosis. Hepatogastroenterology 2000;47:746–750.
17. Morgado PJ Jr, Wexner SD, James K, Nogueras JJ, Jagelman DG. Ileal pouch–anal anastomosis: Is preoperative anal manometry predictive of postoperative functional outcome? Dis Colon Rectum 1994;37:224–228.
18. Ko CY, Rusin LC, Schoetz DJ Jr, Moreau L, Coller JA, Murray JJ, Roberts PL, Arnell TD. Does better functional result equate with better quality of life? Implications for surgical treatment in familial adenomatous polyposis. Dis Colon Rectum 2000; 43:829–837.
19. Becker JM. Anal sphincter function after colectomy, mucosal proctectomy and endorectal ileaoanal pull-through. Arch Surg 1984;119:526–531.
20. Church JM, Saad R, Schroeder T, Fazio VW, Lavery IC, Oakley JR, Milsom JW, Tuckson W. Predicting the functional result of anastomoses to the anus: The paradox of preoperative anal resting pressure. Dis Colon Rectum 1993; 36:895–900.

Discussion

Dr. B.A. Orkin (Washington, D.C.): The question I have is, did you differentiate between the type of ileoanal anastomosis in these patients? Were they all stapled, were they sewn, and did you notice a difference, because I think that has been shown in the past?

Dr. A. Halverson: Yes, we did notice a difference between patients who had a stapled anastomosis and patients who underwent a mucosectomy. The ability of the resting sphincter pressures to predict outcome was similar whether the patient had a stapled or a hand-sewn anastomosis. We did find, as previous studies have shown, that patients with a stapled anastomosis had decreased seepage and decreased incontinence compared to patients with a hand-sewn anastomosis. These data are being written up in a separate report that compares our long-term outcome in patients with stapled vs. hand-sewn anastomoses. That is why I did not specifically present those data here.

Dr. Orkin: So should we use these data to differentiate patients who should have a pouch or are we just going to caution them?

Dr. Halverson: There are two important advantages to identifying patients who have a low resting pressure: (1) managing patients' expectations is one consideration, and sometimes, in patients who have low resting pressures, we

will teach them to do Kegel exercises to try and improve their sphincter tone so that when they have their loop ileostomy closed they have a better outcome. Managing patients' expectations plays a big role in how they interpret the success of their operations.

Dr. K.A. Kelly (Scottsdale, AZ): I have one comment and one question. I notice that the anal squeeze pressures you recorded after the ileal pouch—anal canal operation at the time of ileostomy closure were lower than those before the operation. We have also measured anal squeeze pressures, but we did this months and years after ileostomy closure. We found that these pressures were actually increased compared to before the operation. I believe that the discrepancy rests with the timing of the recordings. We believe that as our patients recover from surgery and start using their sphincters again, the striated muscle of the anal canal actually becomes hypertrophied. A stronger squeeze pressure then results.

My question relates to the fact that 40% to 50% of your patients had fecal incontinence after surgery, a figure that is higher than most of us have reported. It may have to do with how one defines incontinence. Could you please let us know how you did so?

Dr. Halverson: Sure. I think if you go back and study our graph of incontinence, and if you review the literature, the general range reported for incontinence following IPAA is around 25% to 30%, and that is for all comers.

You will note that this is broken up into two groups. So in our patients who have normal resting pressure, the incontinence is less than 30%, which fits in with the reported data in the literature, and this group of patients with low resting pressure is the group that has the higher rate of incontinence. If you take the whole group and put their data together, it is consistent with the rest of the literature.

One of the big problems in assessing incontinence is that every time someone presents their findings on outcome of incontinence, they use a different grading system for incontinence. There is no uniform incontinence scale. That is a problem.

Invited Discussion—Expert Commentator

Keith A. Kelly, M.D. (Scottsdale, AZ): Dr. Halverson's paper showed clearly that preserving the anal sphincter does improve the quality of life after operation in patients undergoing the ileal pouch-anal canal procedure. However, there is kind of a tradeoff here. The best way to protect the sphincter is not to get near it or touch it. Yet, if we do that by using a double staple anastomosis of pouch to distal rectum, we leave diseased mucosa behind in the proximal anal canal. However, if we remove all of the diseased mucosa, including that of the proximal anal canal, we run the risk of damaging the sphincter. We struggle with this at Mayo, and I don't know that we have the right answer. We have tended to favor resecting all the diseased mucosa, but do recognize that some sphincter damage occurs with this.

One Hundred Consecutive Cases of Sentinel Lymph Node Mapping in Early Colorectal Carcinoma: Detection of Missed Micrometastases

Thomas F. Wood, M.D., Dean T. Nora, M.D., Donald L. Morton, M.D., F.A.C.S., Roderick R. Turner, M.D., Decio Rangel, M.D., F.A.C.S., William Hutchinson, M.D., F.A.C.S., Anton J. Bilchik, M.D., Ph.D., F.A.C.S.

Almost one third of patients with "node-negative" colorectal carcinoma (CRC) develop systemic disease. This implies that these patients have occult disease that is inadequately treated by surgery alone. We have coupled sentinel lymph node mapping and a focused pathologic examination to detect occult nodal micrometastases in CRC. Since 1996, sentinel lymph node mapping has been performed in 100 consecutive patients undergoing colectomy for CRC. Peritumoral injection of 0.5 to 1.0 ml of isosulfan blue dye was performed to demonstrate the sentinel node(s). All lymph nodes in the resection specimen were examined by routine hematoxylin and eosin staining. In addition, a focused examination of multiple sections of the sentinel nodes was performed using both hematoxylin and eosin and cytokeratin immunohistochemical analysis (CK-IHC). Overall, lymphatic mapping successfully demonstrated one to four sentinel lymph nodes in 97 (97%) of 100 patients. These sentinel nodes accurately reflected the status of the nodal basin in 92 (95%) of 97 patients. All five of the false negative cases occurred in T3/T4 tumors, and three of the five occurred during the first 30 cases in the experience. Unexpected lymphatic drainage was encountered in eight patients (8%) and altered the operative approach. Twenty-six patients were node positive by routine hematoxylin and eosin staining. Of the remaining 74 patients with hematoxylin and eosin–negative nodes, an additional 18 patients (24%) were upstaged by identification of occult nodal micrometastases that were missed on routine hematoxylin and eosin staining but detected on multiple sections (n = 5) or by CK-IHC (n = 13). The sentinel lymph nodes were the only positive nodes in 19 cases. Sentinel lymph node mapping may be performed in CRC with a high degree of success and accuracy. A focused pathologic examination of the sentinel node detects micrometastatic disease that is missed by conventional techniques in a significant proportion of patients with early CRC. Further studies are necessary to elucidate the clinical relevance of these micrometastases. (J GASTROINTEST SURG 2002;6:322–330.)

KEY WORDS: Colorectal carcinoma, sentinel node, lymphatic mapping, staging

The presence of lymph node metastasis is recognized as the most important prognostic factor in colorectal cancer (CRC). Besides its importance in determining prognosis, the lymph node status is also used as the primary indicator of systemic disease spread and the rationale for postoperative adjuvant chemotherapy. Still, approximately 30% of patients diagnosed with early CRC (American Joint Committee on Cancer [AJCC] stage I or II) develop systemic disease. This implies that this subgroup of patients with early CRC harbors a minimal but significant amount of occult disease that is not detected by current techniques.

Ultrastaging techniques such as multiple-level sectioning, immunohistochemical staining, and polymerase chain reaction assays demonstrate the presence of lymph node micrometastases in a significant proportion of patients whose nodes are negative by routine hematoxylin and eosin pathologic staining.[1–7] Al-

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (oral presentation).

From the John Wayne Cancer Institute (T.F.W., D.T.N., D.L.M., R.R.T., D.R., W.H., A.J.B.), Santa Monica, California.

Supported in part by grant T32 CA 09689 and 090848 from the National Cancer Institute and by funding from the Rogovin-Davidow Foundation, Los Angeles, California and the Rod Fasone Memorial Cancer Fund, Indianapolis, Indiana.

Reprint requests: Anton J. Bilchik, M.D., Ph.D., F.A.C.S., John Wayne Cancer Institute, 2200 Santa Monica Blvd., Santa Monica, CA 90404. e-mail: bilchika@jwci.org

Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(02)00013-6

though the etiology of recurrence in these patients is likely multifactorial, several retrospective studies have established a poorer prognosis in those patients harboring nodal micrometastases demonstrated by ultrastaging techniques.[2,4,6] Because ultrastaging techniques are labor intensive and expensive, we have focused ultrastaging techniques on the sentinel lymph nodes. The sentinel nodes are the lymph nodes most likely to contain early metastases.[8] In CRC, lymphatic mapping and the sentinel lymph node technique are used strictly to improve staging by means of a focused pathologic ultrastaging examination of the sentinel nodes (the lymph nodes most likely to contain the earliest metastases) and not to limit the extent of lymphadenectomy as in melanoma and breast cancer.

We have previously reported our early experience with sentinel lymph node mapping in CRC at two separate institutions[9,10] and demonstrated the feasibility of different technical approaches.[11] The current series updates our experience in our first 100 consecutive cases of sentinel lymph node mapping in CRC performed at the John Wayne Cancer Institute and discusses important lessons learned during this experience.

METHODS

Between August 1996 and November 2000, a total of 100 consecutive patients undergoing resection for clinically localized CRC at the John Wayne Cancer Institute were enrolled in a study of sentinel lymph node mapping. Informed consent was obtained preoperatively from the patients in accordance with an investigational protocol approved by the Institutional Review Board of Saint John's Health Center, Santa Monica, California. Our operative and pathologic approaches were previously described.[10–12] Most of the patients were approached via conventional open colon resection techniques. Recently we have selectively applied a laparoscopic-assisted technique in patients preoperatively deemed to have small early tumors amenable to a laparoscopic-assisted approach.

Open Colon Resection Technique

Laparotomy and routine operative exploration of the abdomen were performed. After resectability had been determined, the involved segment of colon was mobilized. One milliliter of isosulfan blue dye (Lymphazurin, United States Surgical Corp., Ben Venue Laboratories, Inc., Bedford, Ohio) was carefully injected subserosally into four quadrants around the periphery of the tumor using a tuberculin syringe. The dye traveled from the injection site along the lymphatic vessels to the sentinel lymph node(s) typically within 30 to 60 seconds (Fig. 1). Occasionally, gentle dissection of the mesentery was performed to trace the lymphatic path to the sentinel node. Each blue-stained node was marked with a suture, and colectomy was performed in the standard fashion, including all blue nodes in the mesenteric resection specimen. The specimen was submitted for pathologic review.

Laparoscopic Colon Resection Technique

A laparoscopic approach was pursued in those patients interested in such an approach, who preoperatively were deemed to have small, early tumors. Patients were placed on the operating table in the low lithotomy position. Laparoscopic exploration of the abdomen was performed to confirm suitability for laparoscopic colon resection and operative ports were placed according to the specific procedure planned. The appropriate segment of bowel was mobilized by sharp dissection of the peritoneal attachments. If the colonic tumor had not been preoperatively tattooed and was not directly visible via laparoscopy, intraoperative colonoscopy was performed. The tumor or polypectomy site was visualized intraluminally via colonoscope. The site was injected circumferentially in the submucosa with 1 ml of blue dye through the colonoscope. The injection allowed the tumor site to be visualized by the laparoscope. The appropriate segment of colon and mesentery was exposed and under the magnification of laparoscopy, the blue dye was followed from the primary tumor along the lymphatics to the sentinel node(s). Each sentinel node was marked with a clip or suture. A laparoscopic-assisted bowel resection was then undertaken. A minilaparotomy was performed to deliver the specimen. Any additional sentinel lymph nodes identified were marked with sutures. The bowel resection and anastomosis were performed extracorporeally. All blue nodes and lymphatics were included in the mesenteric resection. The specimen was submitted for pathologic review.

During cases in which the tumor site had been tattooed preoperatively or was visible via laparoscope, the lymphatic mapping injection was performed under laparoscopic visualization by a spinal needle placed percutaneously through the abdominal wall. Again, a total volume of 1 ml of blue dye was injected subserosally in a circumferential pattern around the primary tumor site. The remainder of the mapping and resection technique was as outlined above.

Ex Vivo Technique

The ex vivo technique was used either as a primary lymphatic mapping procedure or secondarily for failed

in vivo attempts at lymphatic mapping. Our technique was modified from that of Wong et al.[13] After completion of the colectomy, the specimen was immediately taken to a side table. Lymphatic mapping was then performed on the specimen ex vivo. One to 2 ml of isosulfan blue dye was injected either subserosally or submucosally around the tumor. The dye was visualized as it traveled from the primary site along the lymphatic channels to the sentinel lymph node(s) within the mesentery. Each sentinel node was marked with a suture. The specimen was submitted for pathologic review.

Histopathologic Protocol

Pathologic review entailed routine microscopic analysis of the tumor, margins, and all lymph nodes via hematoxylin and eosin staining. Lymph nodes were manually dissected from the mesenteric fat. Routinely, no chemical fat clearance methods were used. All identified lymph nodes were bisected, and a single section was examined via hematoxylin and eosin staining. If results were negative, each marked sentinel node was examined by a focused technique originally developed for the examination of sentinel lymph nodes draining primary breast carcinomas.[14] The pathologist bisected or sectioned each sentinel node into slices no thicker than 2 to 3 mm. Paraffin sections, each approximately 4 μm thick, were cut at two levels separated by 200 μm. One section from each level was stained with hematoxylin and eosin and another with CK-IHC. A false negative sentinel lymph node was defined as sentinel node that contained no tumor cells when one or more nonsentinel nodes in the specimen were positive for tumor.

Immunohistochemical Staining. Paraffin sections for CK-IHC were placed on charged slides (Superfrost Plus M6416-plus, Baxter Diagnostics Inc., McGaw Park, IL). A standardized procedure used an automated immunostainer (Ventana ES, Ventana Medical Systems, Tucson, Arizona) with enzyme digestion (protease 1) of tissue sections for 8 minutes and AE-1/AE-3 CK antibody (Dako Corp., Carpinteria, CA) staining (1:200 dilution) for 32 minutes. Diaminobenzidene was the chromogen. Immunohistochemical stains were interpreted according to strict criteria that required strong immunoreactivity combined with microanatomic and cytologic features compatible with CRC.

RESULTS

Patient Demographics

The study group consisted of 49 men and 51 women whose average age was 68 years (range 28 to 97 years). Primary tumors were in the right colon (n = 47), transverse colon (n = 6), left colon (n = 5), sigmoid colon (n = 20), or rectum (n = 22). In vivo lymphatic mapping was undertaken during 79 open colon resections and 15 laparoscopic colon resections. Ex vivo lymphatic mapping was undertaken in 12 cases, six of which were primary procedures. In the other six cases, an ex vivo technique was attempted as a salvage secondary lymphatic mapping procedure after a failed attempt during open colon resection; therefore, 106 lymphatic mapping procedures were undertaken in our 100 patients.

Lymphatic Mapping Success Rates

Overall, lymphatic mapping successfully demonstrated at least one sentinel lymph node in 97 (97%) of 100 cases (Table 1). There were, however, eight patients in whom a primary lymphatic mapping procedure undertaken at open colon resection failed to demonstrate a sentinel node (Fig. 2). All of these failures occurred among the first 50 cases in our series. The first two of these failures occurred before we began using the ex vivo technique as a salvage procedure. Of the remaining six cases, we were able to

Table 1. Success and accuracy rates of lymphatic mapping in 100 consecutive cases of CRC

Approach	n	Mapping successful	Average no. of nodes (range)		Accuracy*
			Total	Sentinel	
In vivo					
Open	79	71 (90%)	15 (3–28)	2 (1–4)	68/71 (96%)
Laparoscopic	15	15 (100%)	12 (2–20)	2 (1–3)	14/15 (93%)
Ex vivo	12	11 (92%)	16 (8–24)	2 (1–3)	10/11 (91%)
Overall	106†	97/100 (97 %)	15 (2–28)	2 (1–4)	92/97 (95%)

*Accuracy correspondence between the sentinel lymph node status (metastasis positive or negative) and the regional lymph node basin status as a whole.

†Overall, 106 lymphatic mapping procedures were performed in the 100 patients. In six patients in whom in vivo mapping failed to map a sentinel node, attempts were via the ex vivo approach. Five of these were salvaged by the ex vivo approach, that is, one or more sentinel lymph nodes were successfully mapped.

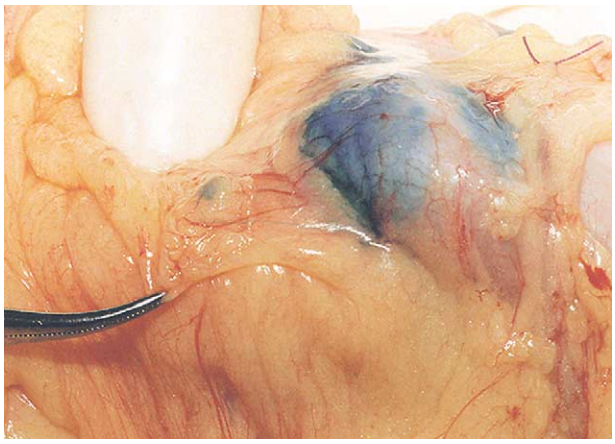


Fig. 1. Shortly after a subserosal injection of isosulfan blue dye, a lymphatic channel can be followed to the sentinel node. Typically, one to four sentinel nodes are mapped during each lymphatic mapping procedure.

salvage five of them by using ex vivo lymphatic mapping to successfully map at least one sentinel node. Interestingly, the single instance in which both a primary and a secondary (ex vivo) lymphatic mapping procedure failed to map a sentinel node occurred in a patient who was found to have 13 of 15 lymph nodes positive for metastases, including nodes replaced with tumor. Two of the three cases in which a sentinel node was never identified occurred in patients with low rectal tumors approached during the first half of our experience. Overall, an average of two sentinel lymph nodes were identified in each case (range 1 to 4), and the average number of nodes harvested from each CRC specimen was 15 (range 2 to 28). There were no failures in identifying at least one sentinel node in our last 50 cases, and no ex vivo salvage mapping procedures were necessary during this period.

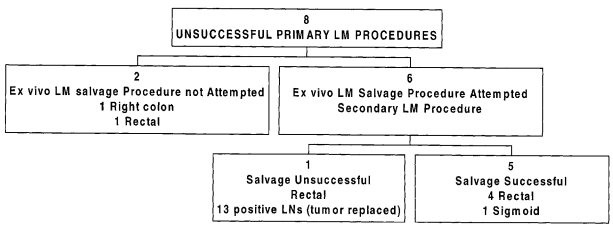


Fig. 2. Among our first 50 cases, we had eight patients in whom our primary attempt at lymphatic mapping (*LM*) was unsuccessful, that is, a sentinel node (*SN*) was not mapped. Six of these failures occurred in rectal tumors. Ex vivo LM was attempted in six patients as a secondary, salvage LM procedure. One or more sentinel nodes were successfully mapped in five of the patients. The one case in which both primary and secondary LM attempts failed was a patient with rectal cancer with extensive nodal and lymphatic disease.

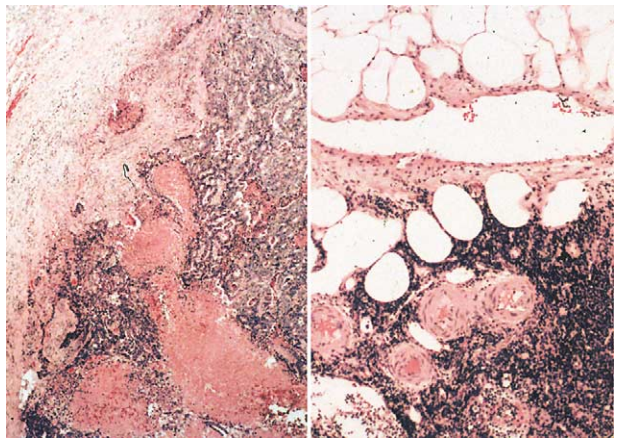


Fig. 3. In this case, a lymph node that was replaced with a tumor likely led to a false negative sentinel node. The photomicrograph on the left demonstrates a mesenteric lymph node that has been extensively replaced with metastatic colon cancer (hematoxylin & eosin stain). The normal nodal architecture has been distorted and the subcapsular sinus has been obliterated by the metastatic tumor. Because of the degree of lymphatic involvement with tumor, the blue dye bypassed the true sentinel node, which was replaced with tumor, and flowed to a false negative sentinel node. The normal-appearing nodal architecture of the false negative sentinel node is demonstrated in the photomicrograph on the right.

Aberrant Lymphatic Drainage

In most cases, the sentinel lymph nodes were near the primary tumor. However, we noted some variations in lymphatic drainage during our experience. We detected primary lymphatic drainage to sentinel nodes outside the margins of conventional colon resection in eight cases; we have termed this aberrant lymphatic drainage. In these cases, the operation performed was altered to include the aberrant sentinel nodes within the field of resection. Specifically, in five cases, the sentinel lymph nodes were mapped at a distance from the primary tumor deep at the base of the mesentery, requiring a more extensive mesenteric resection than is normally performed. Although we routinely perform oncologically appropriate mesenteric resections with high ligation of the colic arteries, we do not routinely ligate them flush with the respective mesenteric artery, and we do not routinely ligate the inferior mesenteric artery flush with the aorta for left-sided lesions. In these five cases, however, the sentinel lymph nodes were in fact found adjacent to the mesenteric arteries, outside the margins of our conventional resection.

The three other cases of aberrant drainage involved right colon carcinomas (two cecal and one midascending colon) in which the sentinel node was mapped to the left of the middle colic vessels. Inter-

estingly, in two of these cases, the aberrantly mapped sentinel lymph nodes were the only positive nodes in the specimen. In one of these cases, the metastases were detected only by CK-IHC. However, in the second case, the metastases were large enough to be detected by routine hematoxylin and eosin staining.

Sentinel Node Accuracy Rates

Identified sentinel lymph nodes accurately reflected the status of the nodal basin in 92 (95%) of 97 patients (see Table 1). All five false negative cases occurred in T3 and T4 tumors, and three of these occurred during the first 30 cases of the experience. A critical review of the false negative cases revealed possible technical explanations for four of the five failures. In two of these cases, the hematoxylin and eosin–positive nodes were replaced with tumor (Fig. 3). In another case, the only "positive" node was a nonsentinel node involved with tumor by direct extension (T4). This case could be arguably classified as negative for nodal metastasis. In the fourth case, a technical error was made during percutaneous injection of the blue dye at laparoscopic-assisted left colon resection. In this patient, a preoperatively placed colonoscopic tattoo with carbon dye was inadvertently placed in an area 2 to 3 cm from the tumor itself. The tumor was not visible laparoscopically so the blue dye was injected circumferentially around the tattoo. Although a blue node was found and marked as a sentinel node, it was falsely negative.

Detection of Occult Micrometastases

Twenty-six patients were node positive by routine hematoxylin and eosin staining. Eighteen other patients had evidence of occult micrometastatic disease by a focused pathologic examination of the sentinel lymph nodes, thereby upstaging an additional 24% (18 of 74) to node positive. In five of these patients, micrometastases were detected by hematoxylin and eosin staining of multiple sections, whereas in the remaining 13 patients micrometastases were documented only on sections stained by CK-IHC. As discussed earlier, another patient was upstaged not by the focused pathologic examination, but rather by mapping of an aberrant sentinel lymph node that was found to be positive by routine hematoxylin and eosin staining. This node would not have been resected and analyzed had the lymphatic mapping procedure not been performed.

Increasing tumor (T) stage was related inversely to the probability of isolated metastases in the sentinel node. In 100% (1 of 1) of node-positive T1 tumors, 86% (6 of 7) of node-positive T2 tumors, 47% (15 of 32) of node-positive T3 tumors, and 0% (0 of 5) of node-positive T4 tumors, the sentinel nodes were the only nodes to contain tumor (Table 2). In the majority of cases in each tumor stage (excluding T4), isolated nodal disease in the sentinel lymph nodes was in the form of micrometastatic disease detected only by a focused pathologic examination (1 of 1 T1, 5 of 6 T2, and 12 of 15 T3 tumors).

DISCUSSION

Standard pathologic methods of harvesting and studying lymph nodes from the resected colon specimens vary from one institution to another. Sampling error may lead to understaging and can occur when informative lymph nodes are not harvested for examination. Special harvesting techniques may reveal small nodes (<5 mm) that are often missed; these small occult nodes can frequently contain metastatic deposits.[15,16] Additionally, routine histologic lymph node examination techniques may also lead to understaging. Routinely, lymph nodes are bisected and only one or two sections are examined by hematoxylin and eosin staining, leaving more than 99% of the node unexamined. However, special pathologic examination of multiple sections of the numerous lymph nodes revealed by these special harvesting techniques is expensive and time consuming. A means of focusing an ultrastaging examination on the lymph nodes most likely to harbor the earliest evidence of metastasis is desirable from both a logistical and economic standpoint.

Application of lymphatic mapping and the sentinel lymph node technique facilitates just such a focused pathologic examination. Unlike in melanoma and breast cancer, the application of the sentinel node technique in CRC is not intended to limit unnecessary lymphadenectomy. Conversely, we have found that in a small number of our patients, the technique actually led us to perform a more aggressive resection that occasionally provided us with important staging information that otherwise would not have been obtained. However, the primary objective of the technique in CRC is to carefully examine the sentinel lymph nodes when there is no evidence of metastases on routine hematoxylin and eosin histologic staining. Because lymph node status remains paramount in staging of CRC, we hypothesize that it is in this patient group that the demonstration of missed micrometastases might shed light on why a disturbing number of patients with "node-negative" CRC go on to have recurrences.

We did not perform CK-IHC on multiple sections of nonsentinel nodes in our current study because of

Table 2. Relationship of tumor stage to lymph node status

Tumor stage	n	Node negative	Node positive	Positive node(s) confined to sentinel lymph node(s)	Cases upstaged by detection of occult micrometastases in sentinel node
T1	25	24	1	1	1
T2	23	16	7	6	5
T3	46	15	31	15	12
T4	6	1	5	0	0
Totals	100	56*	44*	22	18

*Lymph node status after focused pathologic examination. By routine hematoxylin and eosin staining, 26 patients were node positive.

cost and time constraints. Our collaborators at Michigan State University performed CK-IHC on all negative nonsentinel lymph nodes in their first 25 cases. They found that only 0.6% of these initially negative nonsentinel nodes contained evidence of micrometastases. These data demonstrate that the detection of micrometastases in the sentinel lymph node is not solely due to increased histologic sampling.[17]

Our experience with 100 consecutive cases of lymphatic mapping in CRC demonstrates important caveats to keep in mind when applying this technique to CRC. Although the technique is simple with a relatively steep learning curve, we did have problems early in our experience, especially with rectal cancer. We believe that cases of rectal cancer are the most difficult with which to become comfortable. Because the majority of mapping failures early in our experience occurred with rectal cancer (6 of 8) (see Fig. 2), we modified our technique. We now perform a near-complete mobilization of the rectum and mesorectum before injecting the blue dye. This allows observation of the mesorectum and the ability to resect the specimen to more thoroughly examine the mesorectum for the sentinel lymph nodes(s). Cases of rectal cancer, to a greater extent than cases of intraperitoneal colon cancer, often require active dissection of the mesentery to follow the lymphatic channel from the primary tumor site to the sentinel lymph node(s). Although we have not experienced a primary mapping failure in cases of rectal cancer during the second half of our experience, the ex vivo mapping technique is especially advantageous in rectal cancer and may be used as either an accurate primary or salvage lymphatic mapping procedure. Our experience has also demonstrated that a heavy lymphatic and nodal tumor burden may affect both the success and accuracy of the technique. The only case in which we were unable to localize a sentinel node after both primary and salvage lymphatic mapping attempts was in a case of rectal cancer with extensive nodal disease (13 of 15 nodes positive). Similarly, two of the false negative cases could be explained by tumor replacement of the node and lymphatic vessels leading to the true sentinel node, causing the blue dye to take a path of lesser resistance to a noninvolved node. However, it is important to remember that in these cases of obvious nodal involvement, the technique is unlikely to provide useful staging information.

Preliminary studies have demonstrated the prognostic significance of the molecular detection of tumor DNA or RNA in the blood or bone marrow of patients with colon cancer.[18–20] Other investigators have hypothesized that molecular tumor genotyping will someday provide important prognostic and clinically useful information.[21] Although these technologically advanced and specialized assays are likely to be extremely informative in the future, currently they are not generally available and reproducible in a clinical setting. At present, lymph node status remains the single most important and practical prognostic factor in CRC.

Although the prognostic importance of nodal status by routine hematoxylin and eosin staging has been proved, the prognostic significance of nodal micrometastatic disease remains unclear. Whereas a number of small retrospective studies have failed to demonstrate a difference in survival between those with micrometastatic disease and those with node-negative disease,[1,3] other similarly small studies have demonstrated a significant survival advantage in those patients without evidence of micrometastatic disease.[2,4,6] It is possible that the negative studies may have been problematic because of sampling error. In our current series, we have used a focused pathologic examination of the sentinel lymph node(s) to detect micrometastases in a significant number (24%) of patients with hematoxylin and eosin–negative lymph nodes. A prospective trial that includes a standardized focused examination of the sentinel lymph node seems the method most likely to accurately separate those with early metastatic disease from those who are truly node negative and to determine the prognostic value of nodal micrometastases in CRC.

Although a significant proportion of patients with early-stage (AJCC stage I or II) CRC develop systemic disease, treatment of this group of patients

with chemotherapy is currently controversial. To date, no prospective randomized trials have specifically addressed whether chemotherapy provides a survival advantage over surgery alone in stage I or stage II colon cancer. Two published meta-analyses have attempted to provide insight into this question by pooling patients with Duke's stage B colon cancer from available adjuvant therapy trials. In the United States, the National Surgical Adjuvant Breast and Bowel Project (NSABP) analyzed patients with Duke's B colon cancer included in its C-01, C-02, C-03, and C-04 trials.[22] The group concluded that patients with Duke's B colon cancer who are treated with chemotherapy seemed to benefit and that chemotherapy should be routinely considered in these patients. Besides the inherent weakness of this study as a meta-analysis, it has also been criticized because none of its four trials investigated the standard adjuvant regimen of 5-fluorouracil/leucovorin (5-FU/LV) versus surgery alone. A separate international multicenter group recently published the results of a meta-analysis (IMPACT B2) that specifically pooled patients with Duke's B2 colon cancer from five separate trials in which 5-FU/LV was the treatment used.[23] This study found no improvement in event-free or overall survival with adjuvant therapy at more than 5 years' median follow-up. Thus the clinical benefit of adjuvant chemotherapy in early-stage CRC remains unclear.

Notwithstanding, it is likely that certain subgroups of patients with early-stage CRC might indeed benefit from adjuvant therapy. In the future, certain phenotypic and genotypic/molecular characteristics of the primary tumor and lymph nodes might provide a means by which to stratify patient risk of recurrence after resection of early-stage CRC. Just as is currently the case in breast cancer, it is highly probable that future pathologic analyses of CRC specimens will include a panel of both phenotypic and genotypic information important not only for prognosis and systemic chemotherapy considerations, but also for genotypic targeted therapy. Perhaps even more important than determining which patients might benefit from chemotherapy, this information should be crucially important to help determine which patients have an excellent prognosis and definitely should not be treated with chemotherapy. To better elucidate the prognostic significance of phenotypic and genotypic characteristics of nodal micrometastases in CRC and to determine how these factors might play a part in a comprehensive staging schema, we have initiated a prospective trial that is focused on an in-depth analysis of sentinel lymph nodes including immunohistochemical analysis and molecular studies (NCI 1R01CA90848-01, Anton J. Bilchik, M.D., Ph.D., Primary Investigator). This prospective study includes long-term follow-up of patients with nodal micrometastases to determine the natural history of these "earliest metastases."

CONCLUSION

Sentinel lymph node mapping can be performed in carcinoma of the colon and rectum with a high degree of success and accuracy similar to that currently achieved in melanoma and breast cancer. Although the sentinel lymph node technique in CRC is not intended to minimize the extent or morbidity of resection as in melanoma or breast cancer, it facilitates a focused pathologic examination of the lymph nodes most likely to harbor early metastases, the sentinel nodes. This focused examination of the sentinel lymph nodes detects evidence of micrometastatic disease that is missed by conventional techniques in a significant percentage of patients with early-stage CRC. Future studies will determine the prognostic significance of nodal micrometastases in CRC.

REFERENCES

1. Cutait R, Alves VA, Lopes LC, Cutait DE, Borges JL, Singer J, da Silva JH, Goffi FS. Restaging of colorectal cancer based on the identification of lymph node micrometastases through immunoperoxidase staining of CEA and cytokeratins. Dis Colon Rectum 1991;34:917–920.
2. Greenson JK, Isenhart CE, Rice R, Mojzisik C, Houchens D, Martin EW Jr. Identification of occult micrometastases in pericolic lymph nodes of Duke's B colorectal cancer patients using monoclonal antibodies against cytokeratin and CC49. Correlation with long-term survival. Cancer 1994;73:563–569.
3. Jeffers MD, O'Dowd GM, Mulcahy H, Stagg M, O'Donoghue DP, Toner M. The prognostic significance of immunohistochemically detected lymph node micrometastases in colorectal carcinoma. J Pathol 1994;172:183–187.
4. Hayashi N, Ito I, Yanagisawa A, Kato Y, Nakamori S, Imaoka S, Watanabe H, Ogawa M, Nakamura Y. Genetic diagnosis of lymph-node metastasis in colorectal cancer. Lancet 1995;345:1257–1259.
5. Adell G, Boeryd B, Franlund B, Sjodahl R, Hakansson L. Occurrence and prognostic importance of micrometastases in regional lymph nodes in Duke's B colorectal carcinoma: An immunohistochemical study. Eur J Surg 1996;162:637–642.
6. Liefers GJ, Cleton-Jansen AM, van de Velde CJ, Hermans J, van Krieken JH, Cornelisse CJ, Tollenaar RA. Micrometastases and survival in stage II colorectal cancer. N Engl J Med 1998;339:223–228.
7. Calaluce R, Miedema BW, Yesus YW. Micrometastasis in colorectal carcinoma: A review. J Surg Oncol 1998;67:194–202.
8. Morton DL, Wen DR, Wong JH, Economou JS, Cagle LA, Storm FK, Foshag LJ, Cochran AJ. Technical details of intraoperative lymphatic mapping for early stage melanoma. Arch Surg 1992;127:392–399.
9. Saha S, Wiese D, Badin J, Beutler T, Nora D, Ganatra BK,

Desai D, Kaushal S, Nagaraju M, Arora M, Singh T. Technical details of sentinel lymph node mapping in colorectal cancer and its impact on staging. Ann Surg Oncol 2000;7:120–124.
10. Wood TF, Tsioulias GJ, Morton DL, Rangel D, Hutchinson W, Foshag LJ, Turner RR, Bilchik AJ. Focused examination of sentinel lymph nodes upstages early colorectal carcinoma. Am Surg 2000;66:998–1003.
11. Wood TF, Saha S, Morton DL, Tsioulias GJ, Rangel D, Hutchinson W Jr, Foshag LJ, Bilchik AJ. Validation of lymphatic mapping in colorectal cancer: In vivo, ex vivo, and laparoscopic techniques. Ann Surg Oncol 2001;8:150–157.
12. Bilchik AJ, Saha S, Wiese D, Stonecypher JA, Wood TF, Sostrin S, Turner RR, Wang HJ, Morton DL, Hoon DS. Molecular staging of early colon cancer on the basis of sentinel node analysis: A multicenter phase II trial. J Clin Oncol 2001;19:1128–1136.
13. Wong JH, Steineman S, Calderia C, Bowles J, Namiki T. Ex vivo sentinel node mapping in carcinoma of the colon and rectum. Ann Surg 2001;233:515–521.
14. Turner RR, Ollila DW, Stern S, Giuliano AE. Optimal histopathologic examination of the sentinel lymph node for breast carcinoma staging. Am J Surg Pathol 1999;23:263–267.
15. Herrera-Ornelas L, Justiniano J, Castillo N, Petrelli NJ, Stulc JP, Mittelman A. Metastases in small lymph nodes from colon cancer. Arch Surg 1987;122:1253–1256.
16. Koren R, Siegal A, Klein B, Halpern M, Kyzer S, Veltman V, Gal R. Lymph node-revealing solution: Simple new method for detecting minute lymph nodes in colon carcinoma. Dis Colon Rectum 1997;40:407–410.
17. Wiese DA, Saha S, Badin J, Ng PS, Gauthier J, Ahsan A, Yu L. Pathologic evaluation of sentinel lymph nodes in colorectal carcinoma. Arch Pathol Lab Med 2000;124:1759–1763.
18. Soeth E, Vogel I, Roder C, Juhl H, Marxsen J, Kruger U, Henne-Bruns D, Kremer B, Kalthoff H. Comparative analysis of bone marrow and venous blood isolates from gastrointestinal cancer patients for the detection of disseminated tumor cells using reverse transcription PCR. Cancer Res 1997;57:3106–3110.
19. Mori M, Mimori K, Ueo H, Tsuji K, Shiraishi T, Barnard GF, Sugimachi K, Akiyoshi T. Clinical significance of molecular detection of carcinoma cells in lymph nodes and peripheral blood by reverse transcription–polymerase chain reaction in patients with gastrointestinal or breast carcinomas. J Clin Oncol 1998;16:128–132.
20. Yamaguchi K, Takagi Y, Aoki S, Futamura M, Saji S. Significant detection of circulating cancer cells in the blood by reverse transcriptase–polymerase chain reaction during colorectal cancer resection. Ann Surg 2000;232:58–65.
21. Ellis LM. A perspective on sentinel lymph node biopsy in colorectal cancer: The race between surgical technology and molecular oncology. Ann Surg Oncol 2000;7:475–476.
22. Mamounas E, Wieand S, Wolmark N, Bear HD, Atkins JN, Song K, Jones J, Rockette H. Comparative efficacy of adjuvant chemotherapy in patients with Duke's B versus Duke's C colon cancer: Results from four National Surgical Adjuvant Breast and Bowel Project adjuvant studies (C-01, C-02, C-03, and C-04). J Clin Oncol 1999;17:1349–1355.
23. Efficacy of adjuvant fluorouracil and folinic acid in B2 colon cancer. International Multicentre Pooled Analysis of B2 Colon Cancer Trials (IMPAC B2) Investigators. J Clin Oncol 1999;17:1356–1363.

Discussion

Dr. M. Zenilman (Brooklyn, NY): Did you find evidence of any skip lesions? When we started with the breast sentinel node dissections, studies were performed to determine whether or not there were skips of the node by metastatic lesions. Did you see any of that in the colon?

Second, why can't you argue that ultimately this new technique will allow limited colon resections to include the tumor and specific nodal basin rather than continuing to perform formal resections?

Dr. T. Wood: With regard to skip metastasis, when we went back and looked at our five false negative cases, we did not see any that we would specifically refer to as skip metastasis. As I did show, two of the five cases were what might be termed a skip metastasis in that probably the sentinel lymph node completely replaced the lymphatic flow with tumor, and thus skipped around the true sentinel lymph node, going to a nonsentinel lymph node. So if you consider that a skip metastasis, we did see that in two of our five false negative cases.

As far as using the sentinel lymph node technique in colon cancer to minimize the operation, a lymphadenectomy in colon cancer, unlike in breast cancer and melanoma, really adds no morbidity to the procedure. So we are not advocating a change in the criteria for colon resection, except in those few cases where aberrant drainage is found, because, as we have shown, we had two cases where we found positive sentinel lymph nodes outside the field of a normally performed colon cancer operation.

Invited Discussion—Expert Commentator

Keith A. Kelly, M.D. (Scottsdale, AZ): Dr. Wood's presentation on the use of the sentinel lymph node in colon cancer brought up a number of interesting points. He presented data to show that patients who have micro-metastases in their sentinel lymph nodes actually have a worse prognosis that patients who did not. He clearly pointed out that identifying the sentinel lymph nodes allows the pathologist to do detailed studies on these nodes and find micro-metastases, which might otherwise have been missed.

The other point interesting to me was the fact that "aberrant" lymph pathways were identified using the sentinel lymph node technique. The isosulfan blue did not always

proceed to the regional lymph nodes expected to receive lymph drainage from the tumor site. I suppose if the usual regional lymph nodes were filled with cancer cells, the lymph flow might be blocked through those nodes and then more aberrant pathways would be likely to appear.

However, Dr. Wood did show at least one patient where the usual regional nodes were not blocked by cancer cells and yet the sentinel lymph node was in an abnormal or unexpected location. We have certainly found this at our Clinic in dealing with sentinel lymph nodes in melanoma patients. Thus, the sentinel lymph node technique may direct us to do a more complete removal of lymph nodes for colon cancer than we otherwise would have done.

Association of Time to Recurrence With Thymidylate Synthase and Dihydropyrimidine Dehydrogenase mRNA Expression in Stage II and III Colorectal Cancer

Marko Kornmann, M.D., Karl H. Link, M.D., Inga Galuba, Kati Ott, Wolfgang Schwabe, Ph.D., Peter Häusler, Ph.D., Peter Scholz, Jörn Sträter, M.D., Sucan Polat, M.D., Bernhard Leibl, Erika Kettner, M.D., Christoph Schlichting, M.D., Wolfram Baumann, M.D., Harald Schramm, M.D., Ute Hecker, M.D., Karsten Ridwelski, M.D., Jürgen H. Vogt, M.D., Klaus-Ullrich Zerbian, M.D., Frank Schütze, M.D., Ernst D. Kreuser, M.D., Detlev Behnke, Ph.D., Hans G. Beger, M.D.

Patients with International Union Against Cancer (UICC) stage IIb and III colon cancer and stage II and III rectal cancer may receive adjuvant chemotherapy with 5-fluorouracil (5-FU). High levels of thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) have been associated with resistance to 5-FU in advanced colorectal cancer. The aim of this study was to investigate the association of TS and DPD mRNA levels with recurrence-free survival in patients with colorectal cancer who are receiving adjuvant 5-FU–based chemotherapy. TS and DPD mRNA quantitation was retrospectively performed in primary colorectal cancer specimens from patients receiving adjuvant 5-FU using a reverse transcription–polymerase chain reaction technique. The median TS mRNA level in patients with a recurrence (n = 142) was 0.68, and in patients without a recurrence (n = 206) the median level was 0.80 ($P < 0.01$). Patients with a recurrence who had a low TS level (TS $\leq$ 0.9; n = 102) had a median recurrence-free survival of 18 months (range 3.0 to 54 months), and those with a high TS level (TS $>$ 0.9; n = 40) had a median recurrence-free survival of 11 months (range 1.7 to 53 months; $P = 0.0024$). There was no difference in the median recurrence-free survival of patients with low and high DPD mRNA levels. The TS mRNA level may be a useful marker to predict the time to recurrence in patients with colorectal cancer who are receiving adjuvant 5-FU treatment. (J GASTROINTEST SURG 2002;6:331–337.)

KEY WORDS: Thymidylate synthase, dihydropyrimidine dehydrogenase, adjuvant chemotherapy, 5-fluorouracil

Thymidylate synthase (TS) is a key enzyme for DNA synthesis[1] and is irreversibly blocked by 5-fluorouracil (5-FU) after its conversion to 5-fluorodeoxyuridine monophosphate.[2] Several clinical studies have demonstrated that high TS mRNA or protein levels correlate with resistance to 5-FU in several gastrointestinal malignancies.[3–5] Moreover, the better response rate of low-TS colorectal cancers to palliative treatment with 5-FU/leucovorin was associated with a better prognosis.[6,7]

In addition to TS, dihydropyrimidine dehydrogenase (DPD) may also play a key role in 5-FU resistance. DPD catalyzes the initial and rate-limiting step of 5-FU catabolism. Thus the DPD levels in liver

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–24, 2001 (oral presentation.

From the Departments of General Surgery (M.K., K.H.L., S.P., H.G.B.) and Pathology (J.S.), University of Ulm,Ulm; Department of Medicine (E.K.), Städtisches Klinikum Krankenhaus Altstadt, Magdeburg; Department of Surgery I (C.S.), Diakoniekrankenhaus Rotenburg, Rotenburg; Department of General Surgery (W.B.), Klinikum am Eichert, Göppingen; Department of Surgery (H.S.), Wald-Klinikum Gera; Department of Medicine (U.H.), Ernst-Moritz-Arndt-University, Greifswald; Department of Surgery (K.R.), Otto-von-Juerich University, Magdeburg; Department of Surgery (J.H.V.), St. Vinzenz-Krankenhaus, Hanau; Department of Surgery I (K.-U.Z.), Kreiskrankenhaus, Lüdenscheid; Department of General Surgery (B.L.), Marienhospital Stuttgart, Stuttgart; Department of Surgery (F.S.), Winterberg-Kliniken, Saarbrücken; Department of Hematology and Oncology (E.D.K), Krankenhaus Barmherzige Brüder, Regensburg; and the Oncoscreen Research Institute (I.G., K.O., W.S., P.H., P.S., D.B.), Jena, Germany.

Reprint requests: Hans G. Beger, M.D., Department of General Surgery, University of Ulm, Steinhövelstr. 9, 89075 Ulm, Germany. e-mail: hans.beger@medizin.uni-ulm.de

1091-255X/02/$—see front matter
PII: S1091-255X(02)00018-5

cells and in tumor cells may strongly influence the pharmacologic effect of 5-FU.[8] Several studies have demonstrated that high levels of DPD are associated with resistance to 5-FU in the treatment of gastrointestinal malignancies.[9]

However, the importance of TS and DPD expression for adjuvant 5-FU treatment is still unknown. The aim of the present study was to retrospectively analyze the association between intratumoral TS and DPD mRNA levels and survival in patients who have a recurrence during or after adjuvant 5-FU therapy. We now report that patients with recurring disease who had high TS levels had a shorter time to recurrence than patients with low TS levels.

PATIENTS AND METHODS

Patients and Adjuvant Therapy

Patients with colon and rectal cancer who had undergone curative (R0) primary tumor resection and received subsequent adjuvant chemotherapy were entered into our study. All patients were registered at our study center in our adjuvant multicenter trials FOGT-1 or FOGT-2 (study group of gastrointestinal tumors). Patients received adjuvant treatment when they were classified as having International Union Against Cancer (UICC) stage IIb (T4pN0) or stage III (pT1–4pNpositive) colon cancer or UICC stage II (pT3–4pN0) or stage III (pT1–4pNpositive) rectal cancer with complete (R0) resection.[11] All patients in arm A received 5-FU (450 mg/m^2 for 60 to 120 minutes weekly) with levamisol (3 $\times$ 50 mg/day for 3 days biweekly). In arm B, folinic acid (200 mg/m^2 for 10 minutes weekly) was added before 5-FU to the regimen of arm A, and in arm C subcutaneous interferon-α (6 $\times$ 10^6 immunizing units 3 times weekly) was added to the regimen of arm A.[11] All patients in the FOGT-2 trial received postoperative radiation to the pelvis in a dose of 50.4 Gy. Treatment lasted until week 52 after the operation.

Clinical Evaluation

Recurrence-free survival was computed as the number of days from the start of adjuvant treatment to the time of recurrence at any site, and survival was the number of days from the start of adjuvant treatment to death from any cause or until data from patients who were still alive were evaluated.

Total RNA Extraction From Paraffin-Embedded Tissue

Three 10 μm sections were prepared from primary tumor blocks that contained at least 50% tumor cells. RNA from paraffin-embedded tissue was extracted according to the previously described method[12,13] and transcribed into cDNA.

Thymidylate Synthase and Dihydropyrimidine Dehydrogenase mRNA Quantitation

TS and DPD quantitation was carried out by means of a real-time fluorescence detection method (TaqMan PCR, Applied Biosystems, Foster City, California) based on the recently described procedure.[10,14] Polymerase chain reaction was carried out for each gene of interest, and β-actin was used as an internal reference gene.[15] Relative TS and DPD gene expression values using β-actin as the denominator correlated closely with the TS and DPD protein content.[16,17] Relative gene expression of TS and DPD was determined based on the threshold cycles of each gene in relation to the threshold cycle of the corresponding internal standard β-actin.[11] Primers and polymerase chain reaction conditions used have been described previously.[11]

Statistical Analysis

Statistical analysis was performed using SAS software (SAS Institute, Inc., Cary, North Carolina). Data are shown as median and range, and are graphically arranged with the use of box plots. When indicated, the Mann-Whitney rank-sum test was used. For analysis of the association between recurrence-free survival and TS and DPD levels, Kaplan-Meier plots were drawn. To determine the cutoff points for TS and DPD for recurrence-free survival, the log-rank test was used. A P value <0.05 was taken as the level of significance.

RESULTS

Patients

The aim of this study was to investigate the correlation between TS and DPD levels and recurrence-free survival. Therefore we first retrospectively analyzed the available specimens of all patients from five centers who had participated in the FOGT-1 or FGOT-2 study. Because only about 30% of these patients had a recurrence, we subsequently analyzed primary tumor specimens of patients with a known recurrence from six additional centers in order to increase the number of patients with tumor recurrence. The median follow-up was 32.4 months (range 4.0 to 79.8 months).

Thymidylate Synthase and Dihydropyrimidine Dehydrogenase Determination

Results of TS and DPD quantitation from paraffin-embedded tissue were available for 352 patients. Four patients were excluded from the subsequent anal-

Table 1. Characteristics of patients with and without recurring disease

	Recurrence		No recurrence	
	No.	% Total	No.	% Total
Total	142		206	
Sex				
Female	54	38	73	35
Male	88	62	133	65
Age				
≤50 yr	14	10	21	10
50 < x ≤ 60	54	38	73	35
60 ≤ x ≤ 70	47	33	77	37
>70 yr	27	19	35	17
Site				
Colon	83	58	98	48
Rectum	59	42	108	52
Therapy				
A	46	32	56	27
B	56	39	96	47
C	40	28	54	26
Grading				
I	1	0	3	1
II	136	96	192	93
III/IV	5/0	4	10/1	6
Invasion depth				
T1/T2	0/7	5	3/14	8
T3	125	88	179	87
T4	10	7	10	5
Nodal status				
N0	15	11	36	17
N1	88	62	139	68
N2	39	27	31	15
UICC stage				
II	15	11	36	17
III	127	89	170	83

ysis because the sections used for quantitation originated from liver (n = 3) or skin (n = 1) metastases and not from primary tumors. The characteristics of the remaining 348 patients are presented in Table 1.

Association of Thymidylate Synthase and Dihydropyrimidine Dehydrogenase Expression With Recurrence

With respect to most clinical parameters, there was no significant difference between the cohorts with and without a recurrence (see Table 1). However, patients with a tumor recurrence (n = 142) had a median TS level of 0.68 (range 0.12 to 6.07) and patients without a recurrence (n = 206) had a median TS level of 0.80 (range 0.12 to 7.21, $P < 0.01$; Fig. 1, *A*). There was no significant difference in the DPD levels of patients with or without a recurrence (Fig. 1, *B*).

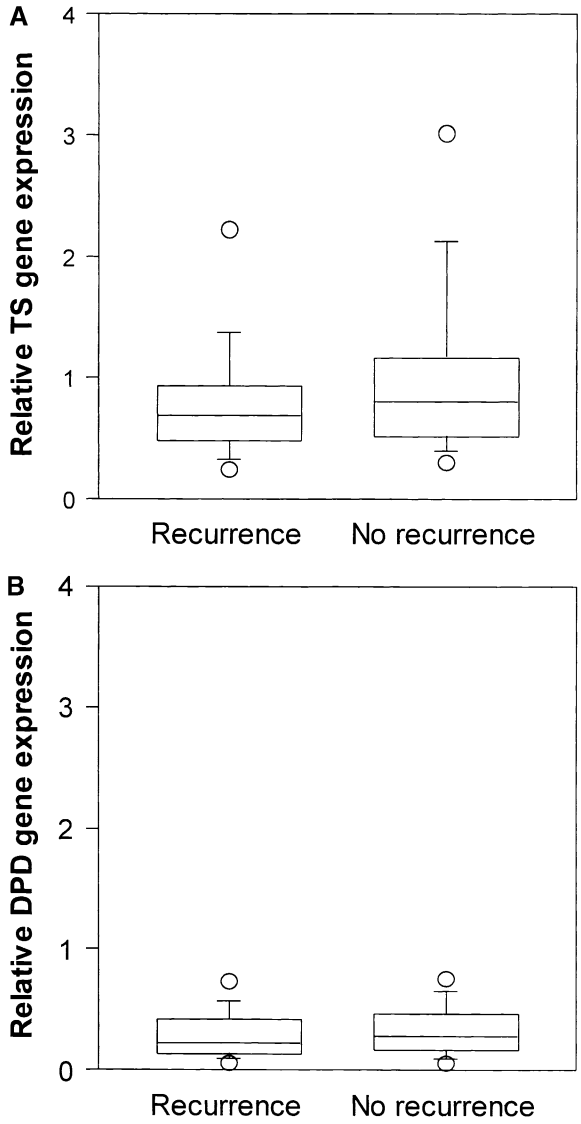


Fig. 1. Box plot analysis of TS and DPD mRNA expression in patients with colorectal cancer who are receiving adjuvant 5-FU chemotherapy comparing patients with (n = 142) and without (n = 206) a recurrence. **A,** TS expression. The median TS level in patients with recurrence was 0.68 (range 0.12 to 6.07) and in patients without recurrence, 0.80 (range 0.12 to 7.21; $P < 0.01$). **B,** DPD expression. The median DPD level in patients with recurrence was 0.23 (range 0.03 to 1.62) and in patients without recurrence, 0.29 (range 0.01 to 1.23). The fifth and ninety-fifth percentile points are indicated as open circles.

Association of Thymidylate Synthase and Dihydropyrimidine Dehydrogenase Levels With Survival in Patients With a Recurrence

As the next step, we investigated the association of TS and DPD levels with survival in patients with recurring disease (n = 142). Patients with a recurrence who had lower TS levels tended to have a longer re-

Table 2. Association of TS and DPD levels with time to recurrence in patients with colorectal cancer who have a recurrence (n = 142) after receiving adjuvant 5-FU chemotherapy

Gene expression	N	Median time to recurrence (mo)	95% Confidence interval
TS ≤0.9	102	17.6*	13.6–21.0 mo
TS >0.9	40	11.2	8.8–12.6 mo
DPD ≤0.4	102	14.5	12.6–18.6 mo
DPD >0.4	40	13.0	12.0–18.1 mo

*P = 0.0024 compared to median value of high TS.

currence-free survival than patients with higher TS levels. Kaplan-Meier curves showed that this difference was most evident at a TS level of 0.9 (log-rank test; P = 0.036). Patients with a TS level ≤0.9 (n = 102) had a median time to recurrence of 18 months (range 3.0 to 54 months), and patients with a TS level >0.9 (n = 40) had a median time to recurrence of 11 months (range 1.6 to 53 months; Table 2). In Fig. 2 the Kaplan-Meier curves for recurrence-free survival are plotted for TS levels ≤0.9 and >0.9. There was no significant difference in the DPD levels of patients with or without recurrence. The Kaplan-Meier curves are shown for patients with a DPD level >0.4 and ≤0.4 for recurrence-free survival (Fig. 3). We did not find a significant correlation between TS or DPD mRNA levels and overall survival in patients with recurring disease.

DISCUSSION

Colorectal cancer is a significant health problem in the Western world.[18] Despite complete clearance (R0 resection) of the primary tumor, approximately 50% of patients develop a recurrence presumably because of the disseminated micrometastases that are present at the time of surgery.[18] Adjuvant treatment with 5-FU/levamisol in colon cancer and 5-FU/levamisol in combination with local irradiation in patients with

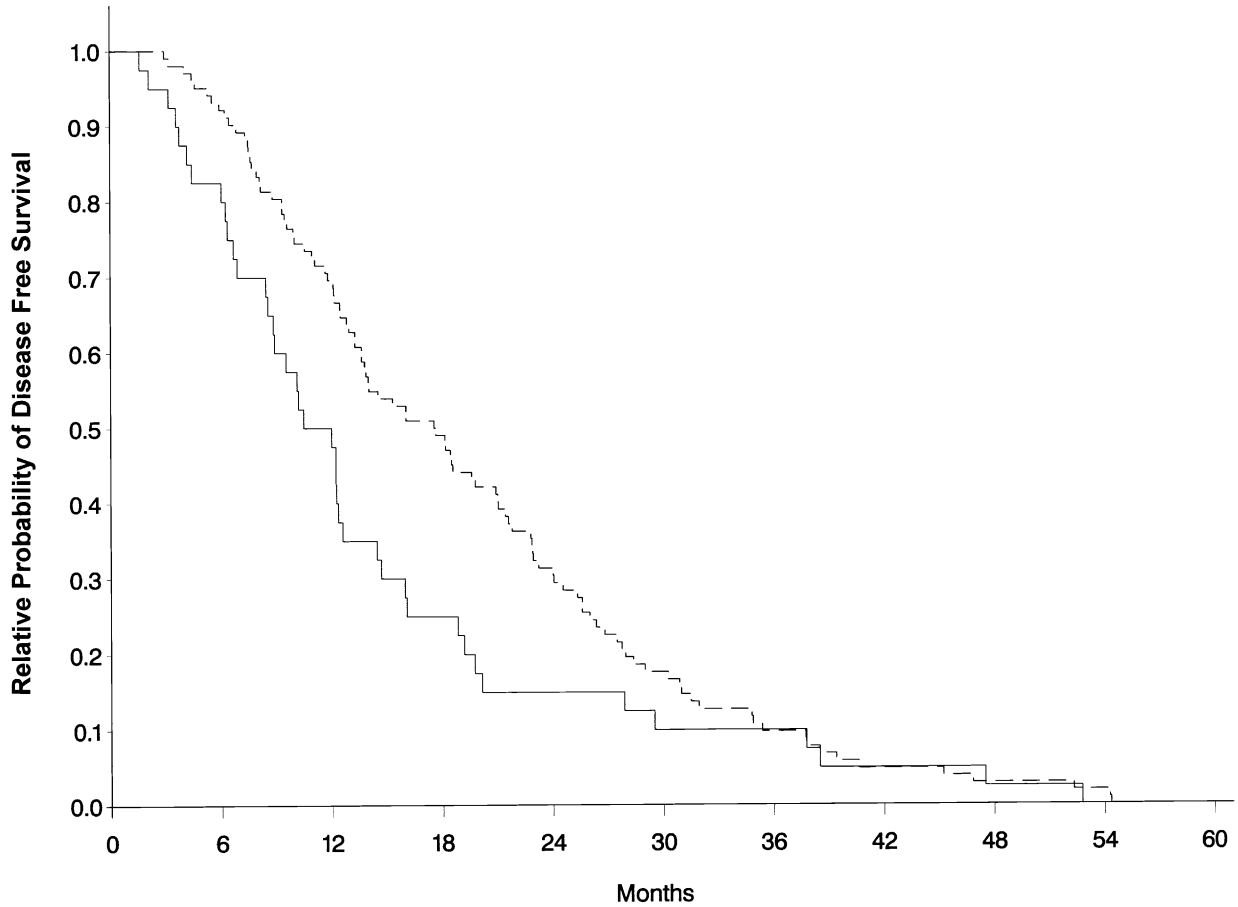


Fig. 2. Kaplan-Meier curves for disease-free survival in patients with recurring disease (n = 142) with low (≤0.9) TS levels (*dashed line*, n = 102) and high (>0.9) TS levels (*solid line*, n = 40). Log-rank test, P = 0.0092.

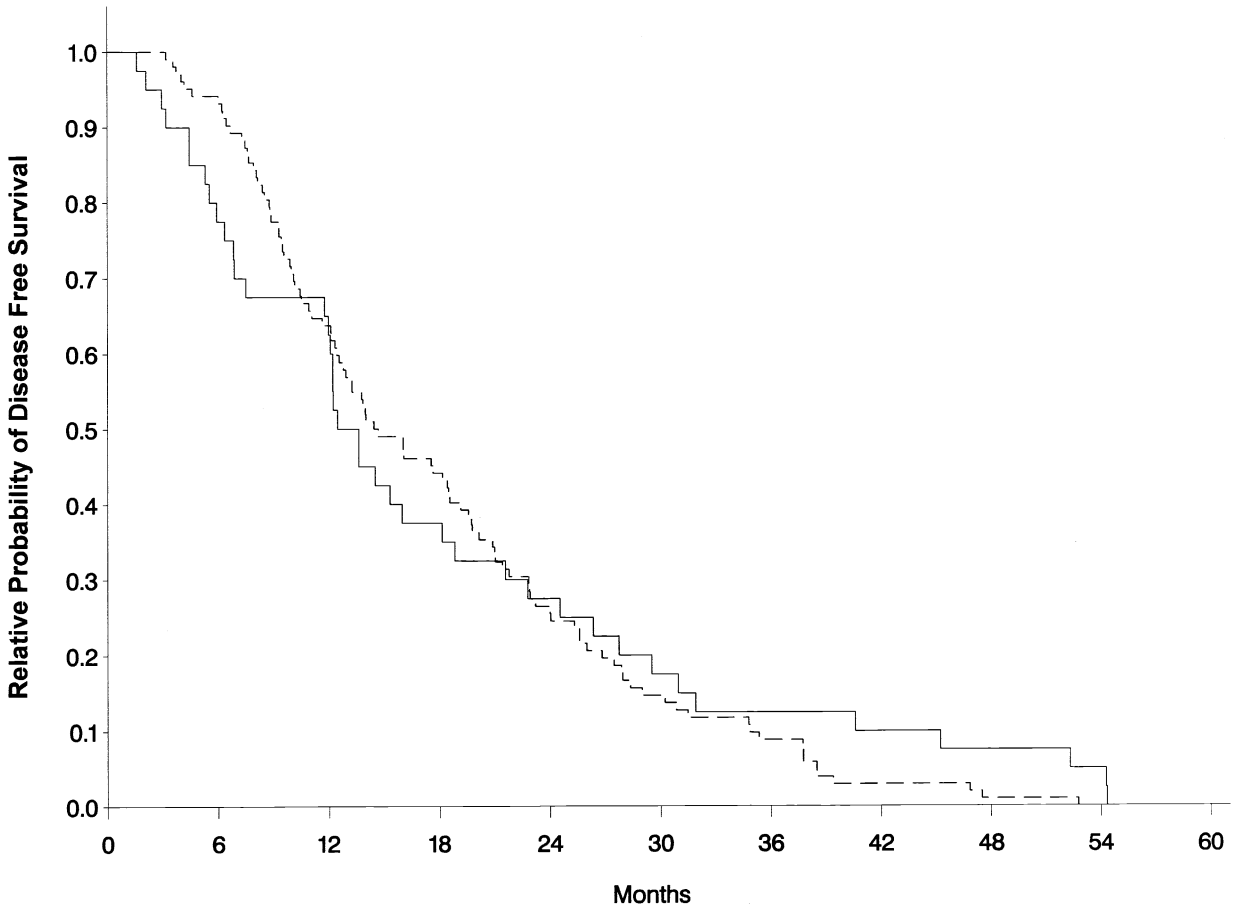


Fig. 3. Kaplan-Meier curves for disease-free survival in patients with recurring disease (n = 142) with low (≤0.4) DPD levels (*dashed line*, n = 102) and high (>0.4) DPD levels (*solid line*, n = 40).

rectal cancer results in a survival benefit. This benefit is attributed to the eradication of circulating cancer cells before they become established.[18] Nonetheless, approximately 30% of patients with UICC stage II and III colorectal cancer who receive adjuvant 5-FU/levamisol treatment still have tumor recurrence. Many of these patients have already had a recurrence of their tumors during the first few months of adjuvant chemotherapy. Obviously these patients do not profit from the adjuvant treatment. Therefore it would be highly desirable to identify patients who are the most likely to benefit from adjuvant 5-FU treatment before the therapy is initiated.

Here we demonstrate, for the first time, that TS expression may be a predictor of chemotherapeutic benefit in adjuvant 5-FU therapy in colorectal cancer. In accordance with previous findings of TS expression and response to 5-FU in advanced colorectal cancer,[6,7,19] we found in our study that disease-free survival was significantly lower in patients with high intratumoral TS levels. It is believed that the 5-FU concentration achievable in vivo results in inefficient TS inhibition when tumoral TS expression is high.[5] Indeed, half of the patients with recurring disease with high TS levels had already developed a detectable recurrence during the 12-month period of adjuvant 5-FU treatment. Although survival of patients who had a recurrence with low TS levels and patients with high TS levels did not differ significantly in comparison to the disease-free survival in the two groups, it is possible that most patients with recurring disease who had high TS levels did not benefit from the 5-FU therapy used in this study to the same extent as patients with a recurrence who had low TS levels. One reason why we only observed a tendency toward longer survival in patients with recurring disease who had low TS levels may be the fact that almost all patients who had a recurrence received second-line treatment (e.g., partial liver or lung resection or different palliative radiochemotherapy regimens, depending on the physician responsible for planning treatment). Therefore the number of patients included in this analysis may be too small to demonstrate a significant influence on survival, regardless of the secondary treatment strategies.

Interestingly, however, our data suggest that patients with high TS levels who receive adjuvant chemotherapy may develop tumor recurrence less frequently than patients with low TS levels. This is in accordance with similar findings based on immunohistochemical analysis and on a small number of patients with colorectal cancer who were given adjuvant 5-FU–based chemotherapy,[16,20] and may be explained by the variation in the behavior of micrometastases compared to established tumors. Taken together, our data on TS expression and recurrence suggest that patients with high TS expression either do not develop recurrences (24% of all patients) or they tend to develop them rather quickly (11% of all patients). On the other hand, patients with low TS expression may tend to have later recurrences but these recurrences are more frequent. After our results are confirmed in prospective studies, these findings may help to individualize and optimize adjuvant treatment regimens.

In addition to TS, DPD may play a role in 5-FU resistance. Unlike several reports which suggested that DPD may also be a strong marker for 5-FU response in the treatment of advanced cancer,[10,21,22] we did not find that DPD had any impact within our adjuvant study cohort. Nevertheless, there may be a trend toward longer disease-free survival in patients with recurring disease who have low DPD mRNA levels. Therefore it may be reasonable in the future to determine DPD levels in addition to TS quantitation.

CONCLUSION

Based on our findings, prospective studies should aim to determine the importance of TS expression in recurrence and survival, and the efficacy of different adjuvant treatment regimens including irinotecan should also be evaluated.[23,24] (Douillard et al.[26]) Eventually such selection strategy could prevent 5-FU toxicity in patients who are unlikely to benefit from 5-FU/folinic acid with or without levamisol and probably would allow quicker access to the optimal drug regimen.

We thank Mrs. U. Kemmer and Mrs. E. Reichert for their secretarial assistance in the FOGT Study Center. We also thank Dr. H. Urban (Department of Pathology, Wald-Klinikum Gera), Dr. C. Quednow (Department of Pathology, Städtischess Klinikum-Krankenhaus Altstadt, Magdeburg), Prof. M. Amthor (Department of Pathology, Diakoniekrankenhaus Rotenburg), Prof. D. Roessner (Department of Pathology, Otto-von-Juerich University, Magdeburg), Dr. S. Blasius and Dr. H. Fitz (Department of Pathology, Klinikum Hanau), Dr. H.F. Tucek (Department of Pathology, Marienhospital, Stuttgart), Prof. G. Lorenz (Department of Pathology, Ernst-Moritz-Arndt-University, Greifswald), Prof. Dr. Mitschke (Department of Pathology, Klinikum Saarbrücken), and Prof. E. Böhm (Department of Pathology, Kreiskrankenhaus Hellersen, Lüdenscheid) for providing paraffin blocks.

REFERENCES

1. Santi DV, McHenry CS, Sommer H. Mechanism of interaction of thymidylate synthetase with 5-fluorodeoxyuridylate. Biochemistry 1974;13:471–481.
2. Danenberg PV. Thymidylate synthetase—A target enzyme in cancer chemotherapy. Biochim Biophys Acta 1977;473:73–92.
3. Lenz HJ, Danenberg KD, Leichman CG, Florentine B, Johnston PG, Groshen S, Zhou L, Xiong YP, Danenberg PV, Leichman LP. p53 and thymidylate synthase expression in untreated stage II colon cancer: Associations with recurrence, survival, and site. Clin Cancer Res 1998;4:1227–1234.
4. Kornmann M, Link KH, Lenz HJ, Pillasch J, Metzger R, Butzer U, Danenberg KD, Danenberg PV, Beger HG. Thymidylate synthase is a predictor for response and resistance in hepatic artery infusion chemotherapy. Cancer Lett 1997;118:29–35.
5. Van Triest B, Pinedo HM, Giaccone G, Peters GJ. Downstream molecular determinants of response to 5-fluorouracil and antifolate thymidylate synthase inhibitors. Ann Oncol 2000;11:385–391.
6. Leichman CG, Lenz HJ, Leichman L, Danenberg K, Baranda J, Groshen S, Danenberg PV. Quantitation of intratumoral thymidylate synthase expression predicts for disseminated colorectal cancer response and resistance to protracted-infusion fluorouracil and weekly leucovorin. J Clin Oncol 1997;15:3223–3229.
7. Cascinu S, Aschele C, Barni S, Debernardis D, Baldo C, Tunesi G, Catalano V, Staccioli MP, Brenna A, Muretto P, Catalano G. Thymidylate synthase protein expression in advanced colon cancer: correlation with the site of metastasis and the clinical response to leucovorin-modulated bolus 5-fluorouracil. Clin Cancer Res 1999;5:1996–1999.
8. Diasio RB, Johnson MR. Dihydropyrimidine dehydrogenase: Its role in 5-fluorouracil clinical toxicity and tumor resistance. Clin Cancer Res 1999;5:2672–2673.
9. Allegra CJ. Dihydropyrimidine dehydrogenase activity: Prognostic partner of 5-fluorouracil? Clin Cancer Res 1999; 5:1947–1949.
10. Salonga D, Danenberg KD, Johnson M, Metzger R, Groshen S, Tsao-Wei DD, Lenz HJ, Leichman CG, Leichman L, Diasio RB, Danenberg PV. Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. Clin Cancer Res 2000;6: 1322–1327.
11. Link KH, Staib L, Bernhart H, Kreuser ED, Suhr P, Röttinger E, Beger HG. Acceptance and toxicity of postoperative adjuvant therapy in colon and rectal cancers. Onkologie 1997; 20:235–238.
12. Woodall CJ, Watt NJ, Clements GB. Simple technique for detecting RNA viruses by PCR in single sections of wax embedded tissue. J Clin Pathol 1993;46:276–277.
13. Masuda N, Ohnishi T, Kawamoto S, Monden M, Okubo K. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucl Acids Res 1999;27:4436–4443.
14. Kornmann M, Danenberg KD, Arber N, Beger HG, Danenberg PV, Korc M. Inhibition of cyclin D1 expression in human pancreatic cancer cells is associated with increased

chemosensitivity and decreased expression of multiple chemoresistance genes. Cancer Res 1999;59:3505–3511.
15. Horikoshi T, Danenberg KD, Stadlbauer TH, Volkenandt M, Shea LC, Aigner K, Gustavsson B, Leichman L, Frosing R, Ray M, Danenberg PV. Quantitation of thymidylate synthase, dihydrofolate reductase, and DT-diaphorase gene expression in human tumors using the polymerase chain reaction. Cancer Res 1992;52:108–116.
16. Johnston PG, Lenz HJ, Leichman CG, Danenberg KD, Allegra CJ, Danenberg PV, Leichman L. Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors. Cancer Res 1995;55:1407–1412.
17. Diasio RB, Danenberg K, Johnson M, Danenberg P. Quantitation of dihydropyrimidine dehydrogenase expression in tumor specimens of patients treated with 5-fluorouracil using a quantitative polymerase chain reaction assay. Proc Am Assoc Cancer Res 1998;39:188.
18. Midgley R, Kerr D. Colorectal cancer. Lancet 1999;353: 391–399.
19. Paradiso A, Simone G, Petroni S, Leone B, Vallejo C, Lacava J, Romero A, Machiavelli M, De Lena M, Allegra CJ, Johnston PG. Thymidylate synthase and p53 primary tumour expression as predictive factors for advanced colorectal cancer patients. Br J Cancer 2000;82:560–567.
20. Takenoue T, Nagawa H, Matsuda K, Fujii S, Nita ME, Hatano K, Kitayama J, Tsuruo T, Muto T. Relation between thymidylate synthase expression and survival in colon carcinoma, and determination of appropriate application f 5-fluorouracil by immunohistochemical method. Ann Surg Oncol 2000;7:193–198.
21. Takabayashi A, Iwata S, Kawai Y, Kanai M, Taki Y, Takechi T, Fukushima M. Dihydropyrimidine dehydrogenase activity and mRNA expression in advanced gastric cancer analyzed in relation to effectiveness of preoperative 5-fluorouracil-based chemotherapy. Int J Oncol 2000;17:889–895.
22. Etienne MC, Cheradame S, Fischel JL, Formento P, Dassonville O, Renee N, Schneider M, Thyss A, Demard F, Milano G. Response to fluorouracil therapy in cancer patients: The role of tumoral dihydropyrimidine dehydrogenase activity. J Clin Oncol 1995;13:1663–1670.
23. Saltz LB, Danenberg K, Paty P, Kelsen D, Kemeny N, Salonga D, Park JM, Danenberg P. High thymidylate synthase activity does not preclude activity of CPT-11 in colorectal cancer (CRC). Proc Am Soc Clin Oncol 1998;17:1080.
24. Saltz LB, Cox JV, Blanke C, Rosen LS, Fehrenbacher L, Moore MJ, Maroun JA, Ackland SP, Locker PK, Pirotta N, Elfring GL, Miller LL. Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. N Engl J Med 2000;343:905–914.
25. Lenz HJ, Leichman CG, Danenberg KD, Danenberg PV, Groshen S, Cohen H, Laine L, Crookes P, Silberman H, Baranda J, Garcia Y, Li J, Leichman L. Thymidylate synthase mRNA level in adenocarcinoma of the stomach: A predictor for primary tumor response and overall survival. J Clin Oncol. 1996;14:176–182.
26. Douillard JY, Cunningham D, Roth AD, Navarro M, James RD, Karasek P, Jandik P, Iveson T, Carmichael J, Alakl M, Gruia G, Awad L, Rougier P. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: A multicentre randomised trial. Lancet 2000;355:1041–1047.

Discussion

Dr. R.A. Hodin (Boston, MA): Do you have a physiologic explanation for the survival in terms of the TS levels?

Dr. H. Kornmann: That is attributed, I think, to the patients with high TS levels who have tumor recurrence; they really have a worse prognosis, but overall, fewer patients with high TS levels have a recurrence. Therefore the overall prognosis is better. But we do not yet know what the relationship is that causes these patients with high TS levels to have fewer recurrences.

Invited Discussion—Expert Commentator

Keith A. Kelly, M.D. (Scottsdale, AZ): Dr. Kornmann's presentation brought to mind that for years we have tested bacteria before treating them with antibiotics to determine the sensitivity of the bacteria to various antibiotics. This has been quite successful. In contrast, using cancer cell cultures to test chemotherapeutic agents for efficacy has not been so successful. Dr. Kornmann's approach looks at specific biochemical functions within cancer cells and then selects anticancer agents on the basis of those biochemical functions. This provocative idea is worthy of pursuit in the future.

Impact of Journal Articles and Grand Rounds on Practice: CT Scanning in Appendicitis

Leigh Neumayer, M.D., M.S., Elizabeth Wako, B.S., Jennifer Fergestaad, M.D., Merril Dayton, M.D.

The purpose of this study was to determine whether a report in a high-impact journal published in January 1998 changed practice patterns and to further explore the impact of a review of the subject in a department of surgery grand rounds (January 2000). Charts from all patients undergoing appendectomy at our institution during three time periods (January to December 1997, January to December 1999, and January to June 2000) were reviewed. Rates of CT scanning, negative appendectomy, and perforated/gangrenous appendicitis were compared for the three periods to determine the impact of the journal article and the subject review during grand rounds on practice patterns and outcomes. Charts from 230 (88%) of 262 patients who underwent appendectomy during the time periods were available for review. Age, percentage of male patients, temperature on admission, and white blood cell count did not differ among the groups. The rate of CT scanning increased significantly from 1997 to 1999 and again in 2000 (6.7%, 43%, and 70%, respectively; $P < 0.001$), whereas the proportion of perforated/gangrenous appendicitis decreased significantly from 33% in 1997 and 31% in 1999 to 13% in 2000 ($P = 0.012$). The use of CT scanning in appendicitis increased both after publication of a report in a high-impact journal and after review during grand rounds. A rate of CT scanning above 45% appeared to affect outcomes as well. (J GASTROINTEST SURG 2002;6:338–341.)

KEY WORDS: Practice patterns, clinical, appendicitis, CT scan, surgical education

Many methods for dissemination of medical information are now available. Investigators rely heavily on peer-reviewed journals for reporting of their results. However, even when results are reported in widely read and cited journals, the impact on practice is not uniform.[1,2] Other more intensive interventions, including concurrent on-site training, have been tried with varying degrees of success.[3–5] Interest generated by a recent report in the literature regarding the usefulness of CT scans in the diagnosis of appendicitis[6] presented an opportunity to explore the impact of journal articles and grand rounds as methods of disseminating information and their impact on practice patterns at our academic institution.

In January 1998, Rao et al.[6] published a study on the use of CT scanning in appendicitis. These investigators reported on a series of 100 consecutive patients with suspected appendicitis who underwent CT scanning preoperatively. CT scans were 98% sensitive and 98% specific in these patients. Thirteen patients were spared a negative appendectomy and 21 patients had an appendectomy earlier in the course of their treatment based on interpretation of their CT scans. In January 2000, a department of surgery grand rounds entitled "Just Appendicitis" was presented. During this presentation the article by Rao et al.,[6] among others, was reviewed. We sought to determine the impact of this article and the subsequent grand rounds on practice and outcomes at our institution.

MATERIAL AND METHODS

Investigators at the University of Utah School of Medicine were presented with a unique situation in which to explore how practice patterns change. The report by Rao et al.[6] was published in January 1998 in the highest impact journal (*The New England Jour-*

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (poster presentation).

From the Department of Veterans Affairs (L.N.), Salt Lake City Health Care System; and Department of Surgery (L.N., E.W., J.F., M.D.), University of Utah School of Medicine, Salt Lake City, Utah.

Reprint requests: Leigh Neumayer, M.D., M.S., Department of Surgery, 50 N. Medical Dr., Salt Lake City, UT 84132. e-mail: leigh.neumayer@hsc.utah.edu

 Published by Elsevier Science Inc.

1091-255X/00/$—see front matter
PII: S1091-255X(01)00088-9

nal of Medicine). In the autumn of 1998, the division of emergency medicine at our hospital discussed the article by Rao et al.[6] at one of their conferences. In January 2000, the department of surgery grand rounds topic was "Just Appendicitis." During this presentation the article by Rao et al., along with other reports on the use of CT scanning in the diagnosis of appendicitis, were reviewed.

To determine the baseline rate of CT scanning, we used information from the calendar year 1997 (the year prior to publication of the Rao study). To determine the impact of the Rao publication and review of it during the emergency medicine conference, we used information from the calendar year 1999. To determine the impact of the department of surgery grand rounds, we used information from January through June 2000. All patients discharged during these periods from the University of Utah Health Care System with the diagnosis of appendicitis, ICD-9 billing code of appendicitis, or CPT code for appendectomy were identified.

CT scanning is immediately available 24 hours a day, 7 days a week. In most cases, scanning is performed as soon as the patient has ingested the oral contrast medium. Patients undergoing CT scanning for appendicitis have their scans performed, in general, approximately 2 hours after they have ingested the oral contrast material. Attending radiologists are available for reading these scans during daylight hours on weekdays; radiology residents are available in house the remainder of the time.

Charts and electronic medical records were reviewed. Demographic data, medical history and physical examination findings, laboratory data, results of preoperative imaging, and pathology reports were all recorded for each patient. It was not possible to determine whether a surgeon or another physician had ordered the laboratory or imaging studies. Length of hospital stay (LOS), both preoperatively and postoperatively, as well as complications were also recorded.

Data were entered into a Statview software system (SAS Institute, Cary, North Carolina) and analyzed using *t*-tests and chi-square analysis for continuous and nominal variables, respectively. To examine the change in the rate of CT scanning over time, data were analyzed by quarter for the time periods studied. A P value <0.05 was considered significant. Approval of the University of Utah Health Sciences Center institutional review board was secured before the study was begun.

RESULTS

A total of 262 patients were identified with the diagnosis of appendicitis during the three time periods. One hundred sixteen patients underwent appendectomy during calendar year 1997, 93 during calendar year 1999, and 53 during the first 6 months of 2000. Charts were available for review for 96 (82.7%) of 116 in 1997, 84 (90.3%) of 93 in 1999, and 50 (94.3%) of 53 in 2000.

Demographic information for patients in the three time periods is presented in Table 1. The rates of preoperative CT scanning during 1997, 1999, and the first 6 months of 2000 were 6.7%, 43.3%, and 70.2%, respectively ($P < 0.001$ between 1997 and 1999 and between 1999 and 2000). These are shown graphically, along with the rates of negative appendectomy and the rates of perforated or gangrenous appendicitis, in Fig. 1. The rates of perforated or gangrenous appendicitis were similar for 1997 and 1999 (33.7% and 30.9%, respectively) but dropped significantly in 2000 (12.5%; $P = 0.012$ compared to 1997 and to 1999). The negative appendectomy rate was not statistically significantly lower, but there was a trend in that direction in 2000 compared to 1997 and 1999 (1997 = 6.3%, 1999 = 6.0%, 2000 = 2.0; P = NS).

The quarterly rate of CT scanning in the study population is illustrated in Fig. 2. The rate of CT scanning in patients with appendicitis did not rise until the second quarter of calendar year 1999, a full year after the publication of the article and several months after review of the information by the emergency room physicians in a conference. Another increase in the rate of CT scanning was apparent after the review during surgery grand rounds in early 2000.

The preoperative LOS illustrated in Fig. 3 for each of the time periods was grouped by whether or not the patient had a preoperative CT scan. In the first 6 months of 2000, the mean preoperative LOS was significantly longer for those patients who underwent CT scanning ($P < 0.05$) but was not longer than the mean preoperative LOS for patients in the two previous time periods, whether or not they had undergone CT scanning. Postoperative LOS was not significantly different between the patients who had preoperative CT scans and those who did not (79.1 $\pm$ 79.6 hours vs. 68.2 $\pm$ 104.3 hours; P = NS).

Table 1. Demographic information

	1997	1999	Jan–June 2000
No. of patients	96	84	50
Mean age (yr)	32.0 ± 14.3	33.0 ± 14.1	31.9 ± 16.2
% Male	49.5	42.9	60.0
Temperature on admission (°C)	37.8 ± 1.0	37.4 ± 0.8	37.3 ± 0.8
White blood cell count (mm^3)	15.0 ± 4.5	14.2 ± 4.0	14.6 ± 4.6

P = not significant for any variable.

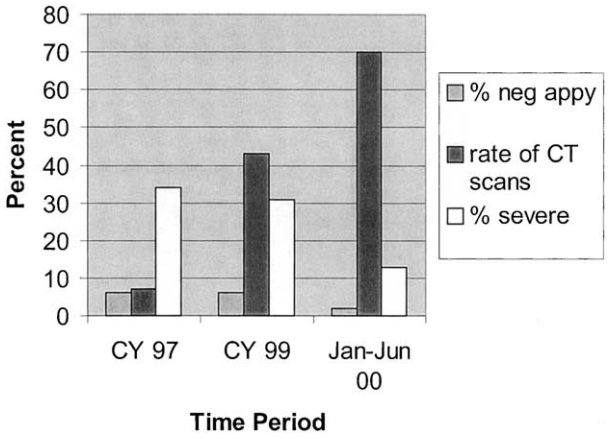


Fig. 1. Rates of negative appendectomies (% *neg appy*), CT scanning, and perforated/gangrenous appendicitis (% *severe*) for the three time periods studied.

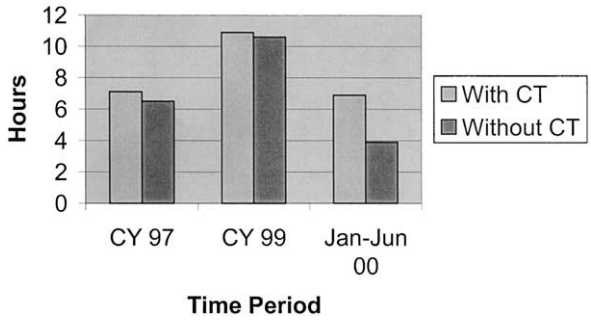


Fig. 3. Preoperative LOS (in hours) for patients with and without CT scans for the three time periods studied. P = not significant between patients with and without CT scans for calendar years 1997 and 1999 (*CY 97 and CY 99*). $P = 0.043$ between patients with and without CT scans for Jan–Jun 00.

DISCUSSION

Whether published studies of new medical and surgical discoveries should or do change the practice patterns of the average clinician is unclear. Some direct and even more indirect evidence exists. Direct evidence includes studies of interventions wherein the outcome measured is a change in the practice pattern. Indirect evidence includes studies using information from large data banks such as tumor registries, hospital discharge registries, and billing information.

Direct evidence-based studies involving surgeons are almost nonexistent. Many studies have been reported that look at interventions to change prescribing practices, referrals to programs, and testing compliance.[3–5] In most of these studies, an "intensive" intervention resulted in increased compliance, whereas no intervention resulted in no change. A multifaceted intervention delivered by nurse practitioners to improve preventive practices in primary care significantly improved performance in the groups that received the intervention.[5] In another intervention study, nurse midwives increased their referrals to a smoking cessation program more than physicians did.[3]

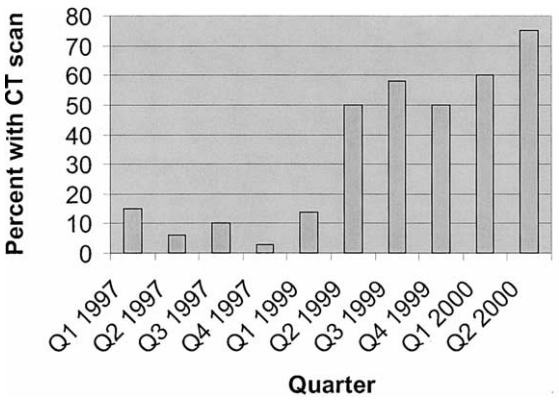


Fig. 2. Rates of CT scanning by quarter for time periods studied.

Peer-reviewed reports of studies appear to have a variable impact on practices. Although physicians have good intentions with regard to incorporating newly reported findings into their practices,[7] and some even report that they have already incorporated these changes, review of charts, billing records, and databases reveal that the rate of change is much lower than what is reported by the physicians themselves.[8]

Minne et al.[9] published the results of a randomized controlled trial at their institution evaluating laparoscopic vs. open appendectomy. They found no significant advantages to laparoscopic appendectomy in the trial. Jones et al.[10] then reviewed charts at that same institution after publication of the study and found that most of the surgeons preferred laparoscopic appendectomy. Seventy-nine percent of appendectomies were started laparoscopically; laparoscopic appendectomies took longer and were more expensive.

Regional differences in adaptation of breast conservation therapy for breast cancer imply that clinicians interpret and incorporate results of randomized controlled trials at varying rates.[1] Geographic region, Medicare fees, sex of the surgeon, and age of the patient have all been shown to be independent predictors of whether or not the patient undergoes breast conservation therapy, although none of these factors correspond to factors presented in published guidelines.[1, 2]

In our study we sought to evaluate the impact of literature and a grand rounds presentation on practice. Although we specifically looked at times related

to the publication by Rao et al.[6] in *The New England Journal of Medicine*, 78 other articles were published in 1997 through 1998 on the subject.[11] In 1999, Lane et al.[12] reported on the high sensitivity and specificity of unenhanced CT scans in the diagnosis of appendicitis. In our study the rate of CT scanning was significantly higher during 1999 compared to 1997, implying a change in practice secondary to the published studies and the review of these studies during a conference attended by the emergency room physicians. However, at the time of the grand rounds presentation in January 2000, the scanning rate had leveled off at approximately 50%. During the grand rounds presentation, the article by Rao et al.,[6] along with others confirming the high sensitivity and specificity of CT scans in appendicitis, were reviewed. In the 6 months after this presentation, the rate of CT scanning increased further. We also found that scanning at a rate of 70% improved outcomes as measured by perforated/gangrenous appendicitis. We have no data to support the reason for this. One hypothesis might be that patients were operated on during their first visit to the emergency room as opposed to having been sent home only to return after perforation.

Flum et al.[13] recently published a population-based analysis of the misdiagnosis of appendicitis and the changes over time. They found that the availability of CT scans and ultrasound images has not decreased the rate of misdiagnosis. Their study period included the years 1987 to 1998. Our data would support their conclusions from that time period. The rate of CT scanning was probably not at a high enough level in the last year of their study (1998) to have an impact on outcomes.

Our study was limited by several factors. First, it was a retrospective review that included only patients who underwent appendectomy. We therefore do not know the number of patients who were referred for CT scanning and then sent home when the CT scan was normal or they were found to have some other pathologic condition. Without that information we could not calculate the sensitivity, specificity, or cost-effectiveness of using CT scans in the diagnosis of appendicitis at our institution. Additionally, it was not possible because of institutional practices to determine who had ordered a CT scan or whether it was ordered with or without a surgeon having seen the patient first. Discussions with the attending physicians in the emergency room revealed that they had reviewed the literature and had begun to incorporate CT scanning into their practices shortly after the publication of the article by Rao et al.[6] Review of the subject at surgery grand rounds further facilitated that incorporation as the surgeons then also understood the data.

CONCLUSION

Publication of important information in highly distributed and widely cited journals may not be the most effective method for bringing about changes in physicians' practices. Our data suggest that oral discussion of important journal articles facilitates changes in practice, as shown by the increase in CT scanning rates after discussion by the emergency room physicians and then a further increase after discussion of the topic at grand rounds. At our institution, performing scans in more than 70% of patients appeared to have a significant impact on the proportion of patients with perforated/gangrenous appendicitis. Institutions desiring to change their practices should use more than publications to communicate these changes and should measure outcomes resulting from the implementation of these changes.

REFERENCES

1. Morrow M, White J, Moughan J, Owen J, Pajack T, Sylvester J, Wilson JF, Winchester D. Factors predicting the use of breast-conserving therapy in stage I and II breast carcinoma. J Clin Oncol 2001;19:2254–2262.
2. Mandelblatt JS, Berg CD, Meropol NJ, Edge SB, Gold K, Hwang YT, Hadley J. Measuring and predicting surgeons' practice styles for breast cancer treatment in older women. Med Care 2001;39:228–242.
3. Cooke M, Mattick RP, Walsh RA. Differential uptake of a smoking cessation programme disseminated to doctors and midwives in antenatal clinics. Addiction 2001;96:495–505.
4. Dieperink ME, Drogemuller L. Industry-sponsored grand rounds and prescribing behavior. JAMA 2001;285:1443–1444.
5. Lemelin J, Hogg W, Baskerville N. Evidence to action: A tailored multifaceted approach to changing family physician practice patterns and improving preventive care. CMAJ 2001;164:757–763.
6. Rao PM, Rhea JT, Novelline RA, Mostafavi AA, McCabe CJ. Effect of computed tomography of the appendix on treatment of patients and use of hospital resources. N Engl J Med 1998;338:141–146.
7. Neill RA, Bowman MA, Wilson JP. Journal article content as a predictor of commitment to change among continuing medical education respondents. J Contin Educ Health Prof 2001;21:40–45.
8. Veninga CC, Denig P, Pont LG, Haaijer-Ruskamp FM. Comparison of indicators assessing the quality of drug prescribing for asthma. Health Serv Res 2001;36:143–161.
9. Minne L, Varner D, Burnell A, Ratzer E, Clark J, Haun W. Laparoscopic versus open appendectomy. Prospective randomized study of outcomes. Arch Surg 1997;132:708–712.
10. Jones B, Ratzer E, Clark J, Zeren F, Haun W. Does peer-reviewed publication change the habits of surgeons? Am J Surg 2000;180:566–569.
11. PubMed literature search using keywords "CT scan" and "appendicitis" for years 1998 and 1999.
12. Lane MJ, Liu DM, Huynh MD, Jeffrey RB Jr, Mindelzun RE, Katz DS. Suspected acute appendicitis: Nonenhanced helical CT in 300 consecutive patients. Radiology 1999;213:341–346.
13. Flum DR, Morris A, Koepsell T, Dellinger EP. Has misdiagnosis of appendicitis decreased over time? A population-based analysis. JAMA 2001;10:286:1748–1753.

Endoscopic Ultrasound–Guided Transrectal Biopsies of Pelvic Tumors

Marco Sailer, M.D., Dieter Bussen, M.D., Martin Fein, M.D., Stephan Freys, M.D., Sebastian E. Debus, M.D., Arnulf Thiede, M.D., Karl-Hermann Fuchs, M.D.

The aim of this study was to evaluate the feasibility, safety, and diagnostic accuracy of endorectal ultrasound–guided biopsies in patients with extrarectal lesions. Data from all patients with suspicious pelvic pathology who underwent endorectal ultrasound–guided biopsies were collected prospectively. To evaluate the accuracy of the diagnosis, all patients with benign histology but primary suspicion of a malignant lesion were followed up for at least 12 months. A total of 48 patients whose median age was 66 years were evaluated. Apart from one postbiopsy hemorrhage, which was managed conservatively, no other complications were encountered. Sufficient tissue was removed to allow histologic examination in all cases. A large variety of diagnoses including primary and secondary malignancies (n = 25) as well as benign pathologies (n = 23) could be established. There were no false positive but three false negative histologies in patients with proven local recurrence of a malignant tumor during the follow-up period. This results in a sensitivity of 88%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 89%. It is concluded that endoscopic ultrasound–guided transrectal biopsy is a safe method with a high diagnostic accuracy in the assessment of pelvic tumors. (J GASTROINTEST SURG 2002;6:342–346.)

KEY WORDS: Endorectal ultrasound, transrectal biopsy, pelvic tumor

Endorectal ultrasound (ERUS) is a well-established imaging technique for the evaluation of anorectal diseases, most notably in the preoperative staging of rectal cancer.[1] Furthermore, ERUS has also gained acceptance in the evaluation of anal sphincter morphology in fecal incontinence,[2,3] in patients presenting with perianal sepsis,[4,5] and in the staging and follow-up of anal carcinoma.[6,7] Because of its high resolution, ERUS is also useful in the assessment of perirectal pathology if the lesions are within the reach of the ultrasound probe, which—depending on the frequency—is generally in the range of 6 to 8 cm.[8] Whereas there is an extensive body of literature dealing with perirectal abscesses[4,8–10] or lymph node involvement in rectal carcinomas,[1,11–14] there are only a few reports of application of ERUS for nonprostatic, nongynecologic pelvic disease in general,[8,9,15,16] and ERUS-guided biopsy of these lesions in particular.[8,17] The aim of this study was, therefore, to evaluate the feasibility, safety, and diagnostic accuracy of ERUS-guided biopsies in patients presenting with extrarectal pelvic lesions.

PATIENTS AND METHODS

During a study period of 7 years, we prospectively collected data from all patients undergoing ERUS at our institution. Patients with suspicious or unclear extrarectal pelvic pathology who underwent ERUS-guided biopsies were included in the present study. After informed consent was obtained, patients were prepared for the procedure with an orthograde bowel lavage and local (i.e., intrarectal) application of an antiseptic solution immediately before the examination. Patients routinely received a single dose of intravenous antibiotic prophylaxis with metronidazole and cefotiam. Sedation with intravenous midazolam was necessary in only three patients.

The procedure was carried out with patients in the lithotomy position using a rotating scanner (Combison 310+, Kretz Co., Zipf, Austria) with a special biopsy aid that allows precise positioning of the biopsy needle under constant ultrasound control. The rectal probe measures 16 cm in length, with a head diameter of 21 mm. During the examination

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (poster presentation).
From the Department of Surgery (M.S., D.B., M.F., S.F., S.E.D., A.T., K.-H.F.), University School of Medicine, Wuerzburg, Germany.
Reprint requests: Marco Sailer, M.D., Department of Surgery, University of Wuerzburg, Josef-Schneider-Str. 2, 97080 Wuerzburg, Germany. e-mail: m.sailer@mail.uni-wuerzburg.de

Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(01)00012-9

the frequency of the bifocal probe can be switched from 7.5 to 5 MHz, thus allowing assessment of extrarectal pathology with a maximum tissue penetration of 7 cm. Resolution for both frequencies is less than 1 mm. The transducer rotates at a speed of 12 cycles per second, generating a 360-degree real-time image. The beams can be emitted longitudinally producing a 100-degree sector or transversely (360-degree image) in reference to the longitudinal axis of the rectum. A special biopsy software program activates a small square in the transverse plane (Fig. 1) and a dotted line in the longitudinal plane (Fig. 2) on the monitor, allowing exact visualization of the biopsy target and needle tract, respectively.

Using the TRU-CUT principle, a high-speed biopsy needle (Magnum, Bard/Angiomed Co., Karlsruhe, Germany) is fired once the target has been precisely visualized, both transversely and longitudinally. The core biopsy needle measures 18 gauge and is 20 cm long, providing a specimen length of 19 mm with a 0.9 mm diameter. All specimens were sent for histologic examination. To evaluate the diagnostic accuracy, all patients with benign histology but primary suspicion of a malignant lesion were followed up for a minimum of 12 months. Sensitivity, specificity, and positive and negative predictive values were calculated.

RESULTS

A total of 48 patients (30 men) who had a median age of 66 years (range 18 to 87 years) underwent ERUS-guided transrectal biopsy. In all patients the indication for the procedure was a suspicion of either metastatic disease (n = 31) or a primary tumor of unknown etiology (n = 17). In almost all cases (91%) there had been additional imaging (CT scan and/or MRI) before the ERUS.

With regard to localization, size, and sonographic appearance of the lesions, a wide range was observed. Tumor localization varied from 4 to 16 cm measured from the anal verge, and the position with regard to the rectal axis was anterior in 19%, posterior in 44%, lateral in 25%, and circular in the remaining 12%. Most lesions (73%) were within 2.0 cm of the rectal wall. The median size of the lesions measured 26 mm (range 7 to 64 mm). Most tumors (61%) were hypoechoic (Fig. 3), whereas an echo-mixed (Fig. 4) or hyperechoic (Fig. 5) pattern was noted in 17% and 22%, respectively. Compared with benign changes, lesions that proved malignant on histopathologic examination appeared hypoechoic more frequently (39% vs. 84%).

Apart from one postbiopsy hemorrhage, which was managed conservatively with no transfusion requirement, no other complications were encountered. Sufficient tissue was removed to allow a histologic examination in all cases. A large variety of diagnoses

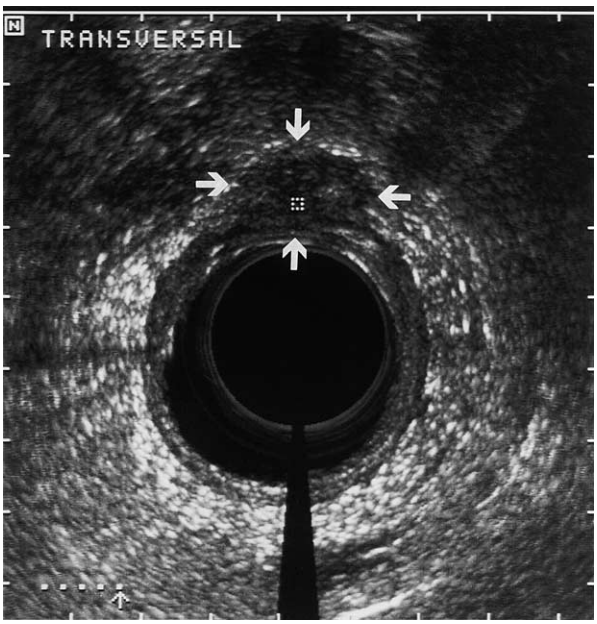


Fig. 1. Hypoechoic extramucosal lesion in the anterior position measuring 20 × 10 mm (*arrows*). The small square indicates the biopsy target. Histologic examination revealed a recurrent adenocarcinoma following low anterior resection for rectal cancer 9 months previously.

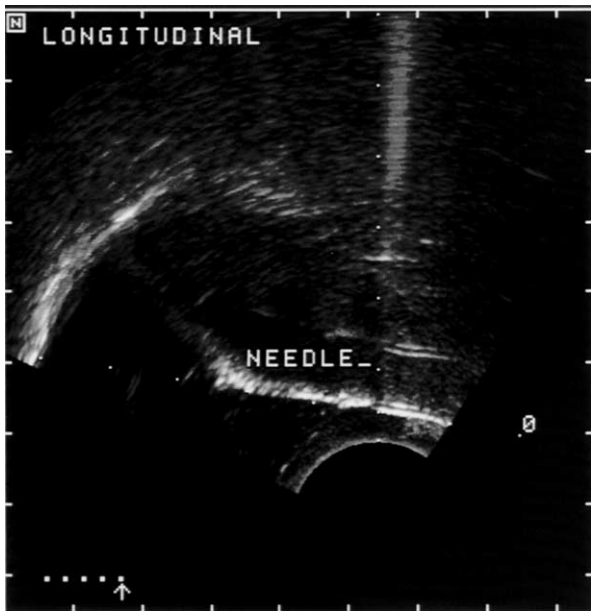


Fig. 2. The hyperechoic biopsy needle is clearly visible in the longitudinal plane. The biopsy tract is along the dotted line. The distance between two dots equals 1 cm.

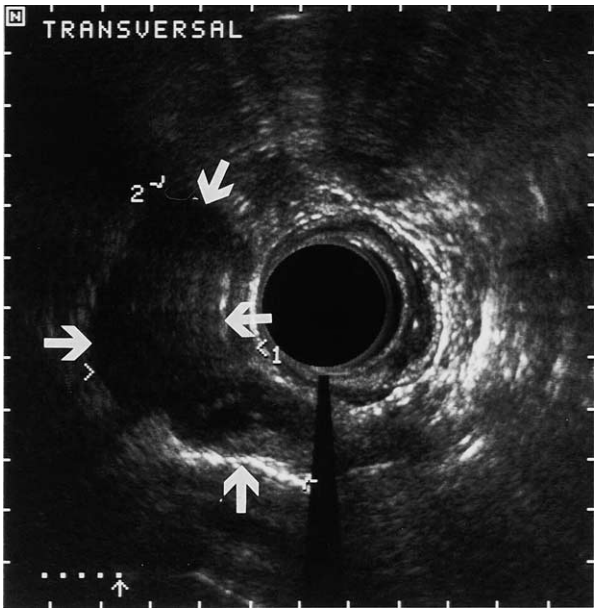


Fig. 3. A large hypoechoic tumor (34 × 65 mm) is seen in the right lateral position localized 12 cm from the anal verge (*arrows*). Histologic examination showed an epidermoid cyst.

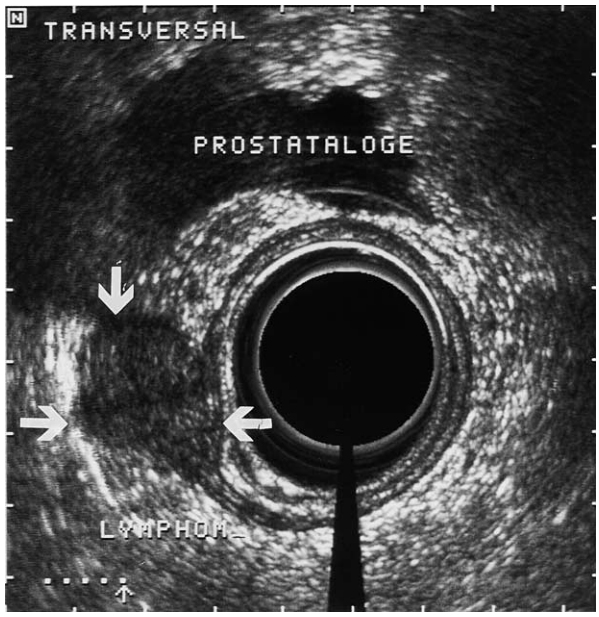


Fig. 5. This 63-year-old patient had undergone radical prostatectomy for cancer 6 months earlier. Anteriorly, the prostatic compartment is seen as an echo-free area. This patient presented with a palpable tumor on rectal digital examination. On transrectal biopsy, this hyperechoic lesion (*arrows*), which measured 47 × 22 mm, proved to be a lymph node metastasis.

including primary and secondary malignancies (Table 1), as well as benign pathologies (Table 2), could be established. There were no false positive but three false negative histologies in patients with proven local recurrence of a malignant tumor during the follow-up period. All three patients were among the first 15 cases in which only one or two aspirations were performed. This results in a sensitivity of 88%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 89%. Subsequently, as a result of these negative biopsies, at least five specimens were removed from every patient. Since then no false negative results have been encountered.

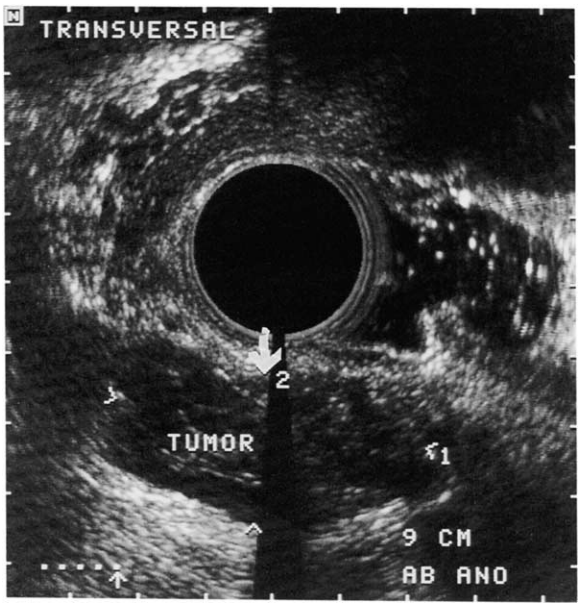


Fig. 4. Transrectal biopsy of this presacral echomixed tumor (45 × 22 mm) disclosed an adenosquamous carcinoma of unknown origin.

DISCUSSION

The diagnostic workup of pelvic tumors can occasionally be laborious. When using imaging techniques such as CT scan and/or MRI in patients with a history

Table 1. Histologic diagnosis of ultrasound-guided transrectal biopsy in patients with malignant histology (n = 25)

Histology	n
Adenocarcinoma	10
Squamous cell carcinoma	5
Prostate cancer	4
Malignant melanoma	2
Adenosquamous carcinoma	1
Signet ring carcinoma	1
Neuroendocrine tumor	1
Undifferentiated carcinoma	1
Total	25

Table 2. Histologic diagnosis of ultrasound-guided transrectal biopsy in patients with benign histology (n = 23)

Histology	n
Connective/fatty tissue	6
Scar tissue	5
Chronic inflammation	4
Nonspecific inflammation	5
Necrosis	1
Leiomyoma	1
Colitis cystica profunda	1
Total	23

of a pelvic malignancy, for example, rectal, prostate, or cervical cancer, it is often difficult to differentiate between scar tissue and recurrent tumor, particularly if the space-occupying lesion is small and tumor markers are only slightly raised if at all. With the advent of positron emission tomography (PET), these lesions can be further divided into those with and those without uptake of the marker used, which is usually 2-[18F] fluoro-2-deoxy-D-glucose.[18] However, to conclusively decide on further therapeutic strategies, which might range from palliative conservative measures to major surgery such as multivisceral resection, a definite histologic diagnosis needs to be established. The same holds true for patients with no previous history who present with a symptomatic or incidental pelvic tumor that cannot be clearly classified by means of either imaging, laboratory investigations, or follow-up examinations, as directed by the respective specialists (i.e., urologist, gynecologist, or colorectal surgeon).

Percutaneous biopsies assisted by either CT or abdominal ultrasound imaging have several limitations. The distance from the surface to the lesion can be considerable, which renders precise puncture difficult if not impossible. Tumors can be obscured by anatomic structures such as the uterus, adnexa, or bowel. Furthermore, patient discomfort and the risk of intraperitoneal infection should also be kept in mind when this type of approach is used. Transrectal ultrasound overcomes most of these restrictions and, most important, spatial resolution is considerably better compared with all other imaging techniques including PET. Therefore ERUS is able to potentially detect lesions at a stage where conventional diagnostic tools would fail to do so. In our series only 13 tumors (27%) were easily palpable by rectal examination. Theoretically these tumors could have been approached by simple digitally guided transrectal needle biopsy. However, ERUS provides biplanar imaging with very precise target localization and needle monitoring. Lesions can be biopsied under visual control rendering the procedure more controlled and consequently safer.

The use of ERUS-guided biopsy of the prostate gland is a well-established method in urology and has gained widespread acceptance in routine practice.[19] Well-documented studies including several thousand patients have shown the safety and efficacy of this procedure with little patient discomfort and very low morbidity.[20] Infection, albeit a rare event, is probably the most serious complication and the administration of a prophylactic antibiotic is generally recommended.[19–21] Because the perirectal tissue is not as well vascularized and contained as the prostate, it was thought that a more thorough preparation, including a complete bowel lavage, was warranted. Furthermore, our present regimen includes a second-generation cephalosporin as well as a potent antianaerobic coverage with metronidazole. Whereas hematuria is a common complication following prostatic needle biopsy and can occur in up to 58%,[22] other hemorrhagic events such as rectal bleeding are generally encountered in only a small percentage.[23] However, rates as high as 37% have been published.[22] As with any other invasive procedure, it seems prudent not to perform ERUS-guided transrectal biopsy in patients taking anticoagulant or thrombolytic medication. If a biopsy is nevertheless needed, medication should either be discontinued, if possible, or the patient should be monitored on an in-patient basis.

There are only a few studies in the nonurologic, nongynecologic literature dealing with transrectal biopsy of pelvic masses. Apart from anecdotal reports of small patient cohorts, [8,16,17,24] there are two articles describing transrectal ultrasound-guided biopsies in larger series of patients with suspected recurrence of rectal carcinoma. Hünerbein et al.[25] report 30 patients who underwent transrectal ERUS-guided biopsies during tumor follow-up. They found 10 patients with malignant histologies, whereas the remaining patients had no evidence of recurrent disease during a follow-up period of 7 months. No complications were encountered. Löhnert et al.[26] used a transperineal approach under local anesthesia in 111 patients with suspicious masses following low anterior resection for rectal cancer. In 52 patients local recurrence was shown on histologic examination. Potentially curative salvage surgery was possible in 31 patients, 25 of whom had been diagnosed by ERUS alone (81% of all curatively resected patients with local recurrence). There were no false positive or negative results. In both of these papers, the total number of biopsies retrieved from each patient is not mentioned. However, taking into consideration our three false negative histologies, all from the early period of the study where only one or two aspirations were performed, we would now recommend obtain-

ing at least five tissue samples per patient. By using this policy we could increase our sensitivity to 100%.

According to the urologic literature, transrectal ultrasound-guided needle biopsy is generally considered to cause minimal or no discomfort and, therefore, it has been common practice to perform this procedure without anesthesia or analgesia.[20,22,27] We can confirm that the vast majority of our patients had only insignificant pain or none at all. We used a sedative drug in only three patients who expressed their anxiety before the biopsy. It appears from the literature that younger patients (i.e., those under 60 years of age) experience a higher level of discomfort, so this subgroup may benefit from analgesia and/or sedation.[20]

CONCLUSION

Endorectal ultrasound is a useful and complementary imaging technique in the assessment of pelvic pathology. Because of its high spatial resolution, ERUS is potentially able to detect lesions at an earlier and therefore more likely curable stage. The major advantage of ERUS, however, is the possibility of performing ultrasound–guided biopsies of suspicious lesions. In the assessment of pelvic tumors, ERUS-guided transrectal needle aspiration is a safe method with minimal morbidity and high diagnostic accuracy.

REFERENCES

1. Kwok H, Bissett IP, Hill GL. Preoperative staging of rectal cancer. Int J Colorectal Dis 2000;15:9–20.
2. Deen KI, Kumar D, Williams JG, Olliff J, Keighley MRB. Anal sphincter defects—Correlation between endoanal ultrasound and surgery. Ann Surg 1993;218:201–205.
3. Eckardt VF, Jung B, Fischer B, Lierse W. Anal endosonography in healthy subjects and patients with idiopathic fecal incontinence. Dis Colon Rectum 1994;37:235–242.
4. Cataldo PA, Senagore A, Luchtefeld MA. Intrarectal ultrasound in the evaluation of perirectal abscesses. Dis Colon Rectum 1993;36:554–558.
5. Deen KI, Williams JG, Hutchinson R, Keighley MRB, Kumar D. Fistulas in ano: Endoanal ultrasonographic assessment assists decision making for surgery. Gut 1994;35:391–394.
6. Goldman S, Norming U, Svensson C, Glimelius B. Transanorectal ultrasonography in the staging of anal epidermoid carcinoma. Int J Colorectal Dis 1991;6:152–157.
7. Herzog U, Boss M, Spichtin HP. Endoanal ultrasonography in the follow-up of anal carcinoma. Surg Endosc 1994; 8:1186–1189.
8. Sailer M, Leppert R, Fuchs KH, Thiede A. Endorectal ultrasound in the evaluation of perirectal disease. Chirurg 1995;66:34–39.
9. Ville EW, Jafri SZ, Madrazo BL, Mezwa DG, Bree RL, Rosenberg BF. Endorectal sonography in the evaluation of rectal and perirectal disease. AJR 1991;157:503–508.
10. Tio TL, Mulder CJ, Wijers OB, Sars PR, Tytgat GN. Endosonography of peri-anal and peri-colorectal fistulas and/or abscess in Crohn's disease. Gastrointest Endosc 1990; 36:331–336.
11. Beynon J, Mortensen NJ, Foy DMA, Channer JL, Rigby H, Virjee J. Preoperative assessment of mesorectal lymph node involvement in rectal cancer. Br J Surg 1989;76:276–279.
12. Glaser F, Schlag P, Herfarth C. Endorectal ultrasonography for the assessment of invasion of rectal tumours and lymph node involvement. Br J Surg 1990;77:883–887.
13. Hildebrandt U, Klein T, Feifel G, Schwarz HP, Koch B, Schmitt RM. Endosonography of pararectal lymoh nodes—In vitro and in vivo evaluation. Dis Colon Rectum 1990; 33:863–868.
14. Milsom JW, Czyrko C, Hull TL, Strong SA, Fazio VW. Preoperative biopsy of pararectal lymph nodes in rectal cancer using endoluminal ultrasonography. Dis Colon Rectum 1994;37:364–368.
15. Milsom JW, Graffner H. Intrarectal ultrasonography in rectal cancer staging and in the evaluation of pelvic disease. Ann Surg 1990;212:602–606.
16. Zainea GG, Lee F, McLeary RD, Siders DB, Thieme ET. Transrectal ultrasonography in the evaluation of rectal and extrarectal disease. Surg Gynecol Obstet 1989;169:153–156.
17. Savander BL, Hamper UM, Sheth S, Ballard RL, Sanders RC. Pelvic masses: Aspiration biopsy with transrectal us guidance. Radiology 1990;176:351–353.
18. Arulampalam THA, Costa DC, Loizidou M, Visvikis D, Ell PJ, Taylor I. Positron emission tomography and colorectal cancer. Br J Surg 2001;88:176–189.
19. Cooner WH, Mosley BR, Rutherford CL, Beard JH, Pond HS, Terry WJ, Igel TC, Kidd DD. Prostate cancer detection in clinical urological practice by ultrasonography, digital rectal examination and prostate specific antigen. J Urol 1990;143:1146–1154.
20. Rodriguez LV, Terris MK. Risks and complications of transrectal ultrasound guided prostate needle biopsy: A prospective study and review of the literature. J Urol 1998;160: 2115–2120.
21. Ashby EC, Rees M, Dowding CH. Prophylaxis against systemic infection after transrectal biopsy for suspected prostatic carcinoma. Br Med J 1978;2:1263–1264.
22. Collins GN, Lloyd SN, Hehir M, McKelvie GB. Multiple transrectal ultrasound-guided prostatic biopsies—True morbidity and patient acceptance. Br J Urol 1993;71:460–463.
23. Rietbergen JB, Kruger AE, Kranse R, Schröder FH. Complications of transrectal ultrasound-guided systematic sextant biopsies of the prostate: Evaluation of complication rates and risk factors within a population-based screening program. Urology 1997;49:875–880.
24. Milsom JW, Lavery IC, Stolfi VM, Czyrko C, Church JM, Oakley JR, Fazio VW. The expanding utility of endoluminal ultrasonography in the management of rectal cancer. Surgery 1992;112:832–841.
25. Hünerbein M, Schlag PM. Three-dimensional endosonography for staging of rectal cancer. Ann Surg 1997;225:432–438.
26. Löhnert MSS, Doniec JM, Henne-Bruns D. Effectiveness of endoluminal sonography in the identification of occult local rectal cancer recurrences. Dis Colon Rectum 2000; 43:483–491.
27. Irani J, Fournier F, Bon D, Gremmo E, Dore B, Aubert J. Patient tolerance of transrectal ultrasound-guided biopsy of the prostate. Br J Urol 1997;79:608–610.

Laparoscopic Antireflux Surgery With Routine Mesh-Hiatoplasty in the Treatment of Gastroesophageal Reflux Disease

Frank A. Granderath, M.D., Ursula M. Schweiger, M.D., Thomas Kamolz, M.D., Martin Pasiut, M.D., Christoph F. Haas, M.D., Rudolph Pointner, M.D.

One of the most frequent complications after laparoscopic antireflux surgery is intrathoracic migration of the wrap ("slipped" Nissen fundoplication). The most common reasons for this are inadequate closure of the crura or disruption of the crural closure. The aim of this prospective study was to evaluate surgical outcomes in patients who underwent laparoscopic antireflux surgery with simple nonabsorbable polypropylene sutures for hiatal closure in comparison to patients who underwent routine mesh-hiatoplasty. Between 1993 and 1998, a group of 361 patients underwent primary laparoscopic Nissen or Toupet fundoplication with the use of simple nonabsorbable polypropylene sutures for hiatal closure. Since December 1998, in all patients (n = 170) who underwent laparoscopic antireflux surgery, a 1 × 3 cm polypropylene mesh was placed on the crura behind the esophagus to reinforce them. Functional outcome, symptoms of gastroesophageal reflux disease, and postoperative complications such as recurrent hiatal hernia with or without intrathoracic migration of the wrap have been used for assessment of outcomes. In the initial series of 361 patients, postoperative herniation of the wrap occurred in 22 patients (6.1%). Of these 22 patients, 17 of them (4.7%) had to undergo laparoscopic redo surgery. The remaining five patients were free of symptoms. In comparison to these results, in a second group of 170 patients there was only one (0.6%) who had postoperative herniation of the wrap into the chest. There have been no significant differences in objective data such as DeMeester scores or lower esophageal sphincter pressure between the two groups. Postoperative dysphagia was increased during the early period after surgery in patients undergoing mesh-hiatoplasty but resolved without any further treatment within the first year after laparoscopic antireflux surgery. We concluded that routine hiatoplasty with the use of a polypropylene mesh is effective in preventing postoperative herniation of the wrap and leads to a significantly better surgical outcome than closure of the hiatal crura with simple sutures, without any additional long-term side effects. (J GASTROINTEST SURG 2002;6:347–353.)

KEY WORDS: Gastroesophageal reflux disease, laparoscopic antireflux surgery, mesh-hiatoplasty

Laparoscopic antireflux surgery (LARS) has replaced the open approach in centers worldwide and has been shown to be an effective alternative to long-term medical treatment for gastroesophageal reflux disease (GERD).[1,2] Several studies have shown that LARS can achieve good to excellent results with minimal morbidity and acceptable mortality when performed by an experienced laparoscopic surgeon.[3,4] However, a number of postoperative complications have been reported.[5,6] The rate of complications after LARS is the same as that reported for open antireflux surgery, but recurrence of hiatal hernia seems more likely after laparoscopic procedures.[7,8] The most common complication after LARS is intrathoracic herniation of the wrap into the chest, which is caused by inadequate closure of the crura or disruption of the crural closure. Typical symptoms of this so-called "slipped" Nissen fundoplication are recurrent or persistent heartburn, dysphagia, or a combination of the two. Depending on the severity of

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (poster presentation); and the Ninth International Congress of the European Association for Endoscopic Surgery, Maastricht, The Netherlands, June 15, 2001; and published as an abstract in *Gastroenterology* 120:A 480, 2001.

From the Department of General Surgery (F.A.G., U.M.S., T.K., M.P., C.F.H., R.P.), Hospital Zell am See, Zell am See, Austria.
Reprint requests: F.A. Granderath, M.D., Department of General Surgery, A. Ö. Krankenhaus Zell am See, A-5700 Zell am See, Austria. e-mail: fagzellamsee@yahoo.com

1091-255X/02/$—see front matter
PII: S1091-255X(01)00025-7

symptoms, the complication can be treated either with medication or by redo surgery. The relatively high frequency of postoperative wrap herniation into the chest in our patients caused us to use a mesh to achieve a more effective closure of the hiatus.

The aim of the present study was to evaluate outcomes in the first group of 170 patients who underwent a "floppy" Nissen or Toupet fundoplication using routine mesh-hiatoplasty in comparison to 361 patients in whom simple nonabsorbable polypropylene sutures were used.

MATERIAL AND METHODS

Since September of 1993, a total of 531 patients (319 men and 212 women), with a mean age of 53 years (range 27 to 78 years), who had symptomatic GERD underwent laparoscopic antireflux surgery in our surgical unit. Laparoscopic floppy Nissen fundoplication was carried out in all patients with normal esophageal motility (n = 376). Patients with poor esophageal motility (<30 mm Hg in the lower esophageal segments in response to wet swallows) or severely disordered peristalsis (>40% simultaneous contractions in wet swallows) underwent laparoscopic Toupet fundoplication (n = 155). All patients had a lengthy history of GERD symptoms (mean 8.1 ± 7.0 years) and had been treated with proton pump inhibitors (20 to 80 mg omeprazole/day) for a mean period of 16.8 months. Basic requirements for LARS in all patients included a precise evaluation of GERD symptoms, esophagogastroduodenoscopy (EGD) with biopsy and histologic examination of the gastroesophageal junction, 24-hour pH monitoring, and esophageal manometry as described previously.[9] Indications for surgery were persistent or recurrent GERD symptoms despite optimal medical treatment, persistent or recurrent complications of GERD, noncompliance with lifelong oral medication, decreased quality of life, and a weak lower esophageal sphincter (LES) pressure (<6 mm Hg).

Two groups of patients were evaluated in this study. The first group included 361 patients (239 men and 122 women), with a mean age of 54.8 years, who underwent laparoscopic antireflux surgery between September 1993 and November 1998 with the use of simple nonabsorbable polypropylene sutures for hiatal closure. Laparoscopic floppy Nissen fundoplication was performed in 233 patients and Toupet fundoplication was used in 128 patients. After December of 1998, a second group of 170 patients (80 men and 90 women), with a mean age of 51.2 years, underwent LARS in which routine polypropylene mesh-hiatoplasty was used for hiatal closure. Laparoscopic floppy Nissen fundoplication was performed in 143 patients, and Toupet fundoplication in 27 patients.

Hiatal Closure Technique

After exposure of the hiatal region, the first step was to dissect both crura exactly, beginning at the right crus with immediate identification of the commissure of the crura. The esophagus then had to be elevated to separate loose tissue behind the esophagus. In this manner, a window was opened behind the esophagus through which the posterior portion of left crus could be identified and mobilized from the left side of the esophagus. At this point the esophagus was mobilized high up intrathoracically to gain length and bring the LES intra-abdominally. Dissection of the esophagus was performed until a 3 to 5 cm segment was exposed intra-abdominally. In the first group of patients undergoing hiatal closure with nonabsorbable polypropylene sutures, the crura were brought together using two, three, or four 2/0 polypropylene sutures for posterior hiatoplasty depending on the size of the hiatal hernia. After suturing of the crura, the esophagus is laying loose in the hiatus (Fig. 1). Calibration has only been used at the beginning of LARS in our surgical unit. In the group undergoing mesh-hiatoplasty, the hiatus has been closed with the use of a 1 × 3 cm polypropylene mesh, which we cut out of a 10 × 15 cm polypropylene mesh (Tyco Healthcare, Vienna, Austria) that is normally used for TAPP hernia repair. After dissection as described previously, the mesh is secured with one stitch on the lateral sides of both the right and left crura (Fig. 2). For large hiatal defects, the mesh has been used in addition to one or two sutures (Figs. 3, and 4).

Follow-Up

In general, postoperative EGD was performed routinely 6 weeks after surgery in our surgical unit or by a local gastroenterologist. Additionally, in all patients a standard postoperative examination was performed at 3 months and at 1 year after surgery, including 24-hour pH monitoring, esophageal manometry, and evaluation of GERD symptoms. Postoperative results in group 1 were evaluated 3 months (group 1: n = 359; group 2: n = 167) and 1 year (group 1: n = 349; group 2: n = 158) after surgery.

In all patients referred to our unit postoperatively with recurrent symptoms, routine diagnostic EGD was followed by a barium swallow. In all patients, the diagnosis of a wrap herniation into the chest was established by use of both procedures.

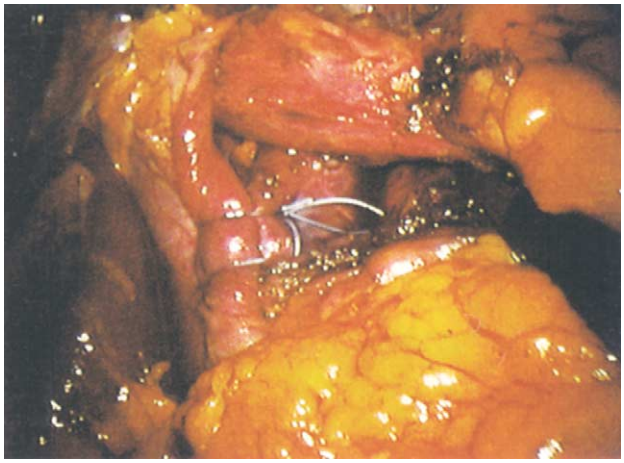


Fig. 1. Intraoperative situs after hiatal closure using two single sutures without mesh as performed in the first group of 361 patients. After suturing of the crura, the esophagus is laying loose in the hiatus.

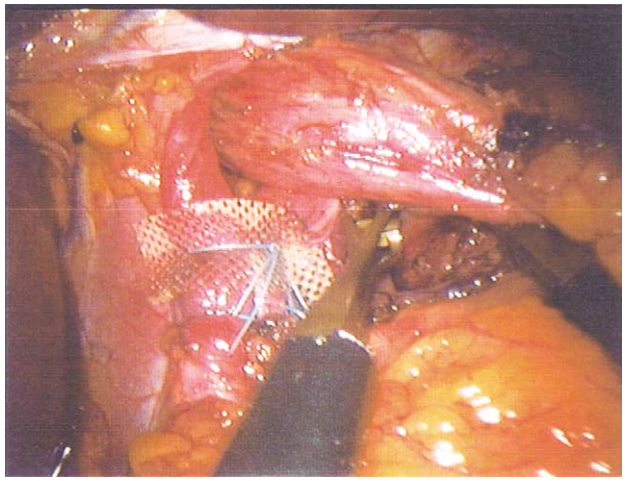


Fig. 3. Completed mesh-hiatoplasty before creation of the Nissen fundoplication.

Symptom Evaluation

In addition to traditional outcome criteria such as DeMeester scores and LES pressure, we evaluated the extent of the following symptoms: heartburn, regurgitation, chest pain, and dysphagia. These symptoms were subjectively evaluated using a simple verbal rating scale. Symptoms were described as follows: none, mild to moderate, or severe.

Statistical Analysis

The SPSS program was used for statistical analysis. Treatment results were analyzed by means of repeated-measures procedures. $P < 0.05$ was considered significant. Data are reported as mean ± standard deviation, range, or percentage.

RESULTS

Traditional Outcome

In group 1, LARS was successfully completed in 359 patients. In two cases conversion to open surgery was necessary because of severe bleeding in the early learning period. The mean LES pressure increased from a preoperative value of 2.6 ± 2.1 mm Hg to 12.4 ± 3.0 mm Hg at 3 months and 13.8 ± 3.6

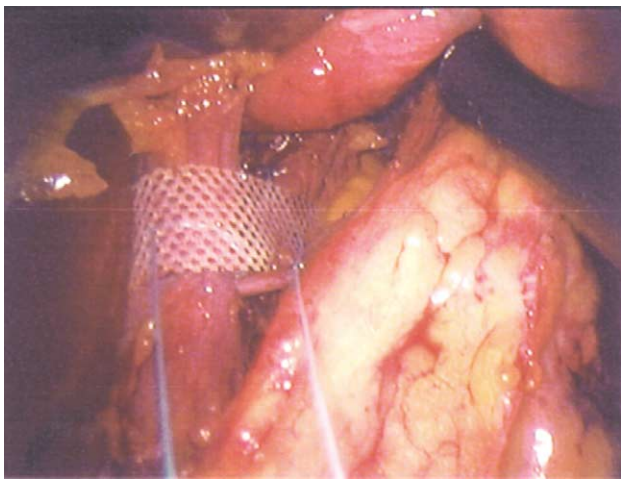


Fig. 2. Mesh-hiatoplasty. The polypropylene mesh (1 × 3 cm) has been brought into the abdomen and affixed with one stitch onto the sides of both the right and left crura.

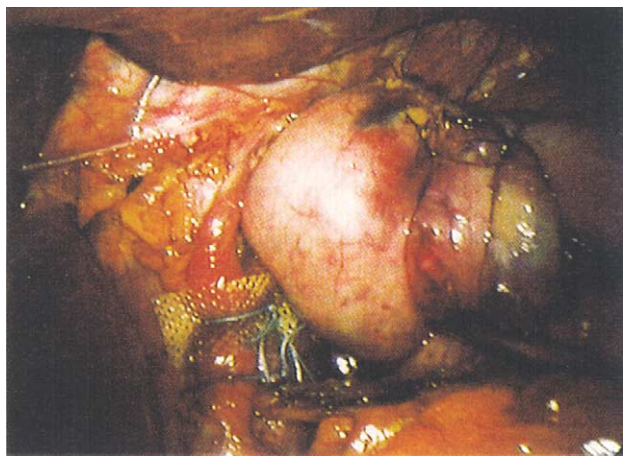


Fig. 4. Mesh-hiatoplasty and 360-degree floppy Nissen fundoplication. The mesh is attached to hiatal crura and does not come in contact with the posterior portion of the esophagus.

Table 1. DeMeester score and lower esophageal sphincter (LES) pressure before and after laparoscopic antireflux surgery (LARS) in group 1 (sutures)

	Before LARS (n = 361)	3 mo after LARS (n = 359)	1 yr after LARS (n = 349)
DeMeester score	64.9 ± 22.0	12.3 ± 11.8	9.6 ± 3.0
LES pressure/mm Hg	2.6 ± 2.1	12.4 ± 3.0	13.8 ± 3.6

mm Hg at 1 year after surgery. The mean DeMeester score decreased from 64.9 ± 22.0 preoperatively to 12.3 ± 2.8 at 3 months and 9.6 ± 3.0 at 1 year postoperatively (Table 1). The mean follow-up for these patients was 4.2 years (range 2.5 to 8.2 years). In all 170 patients in group 2, the procedure was successfully completed laparoscopically. The mean LES pressure increased from a preoperative value of 2.8 ± 1.9 mm Hg to 12.6 ± 2.6 mm Hg at 3 months and 14.0 ± 2.9 mm Hg at 1 year postoperatively. Twenty-four-hour pH monitoring showed a decrease in the mean DeMeester score from 65.1 ± 23.1 preoperatively to 12.5 ± 2.6 at 3 months and 9.8 ± 2.9 at 1 year postoperatively (Table 2).

Mean follow-up for patients in group 2 was 16 months (range 12 to 30 months).

Intrathoracic Wrap Herniation

In the initial group of 361 patients, postoperative wrap herniation into the chest occurred in 22 cases (6.1%). In all 22 patients, a "slipped Nissen" occurred within the first year postoperatively (Table 3). In five patients (1.3%), intrathoracic wrap herniation was diagnosed on routine EGD 6 weeks after surgery. These patients were asymptomatic and did not need any further treatment. Thirteen patients (3.6%) had recurrent reflux and four patients (1.1%) had severe dysphagia postoperatively. These patients underwent laparoscopic redo surgery and are currently free of symptoms. All reoperations were performed successfully by means of the laparoscopic technique.

Table 2. DeMeester score and lower esophageal sphincter (LES) pressure before and after laparoscopic antireflux surgery (LARS) in group 2 (mesh)

	Before LARS (n = 170)	3 mo after LARS (n = 167)	1 yr after LARS (n = 158)
DeMeester score	65.1 ± 23.1	12.5 ± 2.6	9.8 ± 2.9
LES pressure/mm Hg	2.8 ± 1.9	12.6 ± 2.6	14.0 ± 2.9

Table 3. Postoperative wrap herniation (slipped Nissen) 1 year after surgery

	Slipped Nissen
Group 1 (n = 361)	22 (6.1%)
Group 2 (n = 170)	1 (0.6%)

Of the 170 patients in group 2 who underwent routine polypropylene mesh–hiatoplasty, there was only one patient (0.6%) who had postoperative herniation of the wrap into the chest. This patient suffered from acute and severe dysphagia within the first 4 weeks after the initial procedure. This patient underwent laparoscopic redo surgery, which was technically very difficult because of massive adhesions as compared to reoperations in patients where mesh was not used. Intraoperatively we have found that inadequate placement of the mesh is the reason for postoperative wrap migration. After a laparoscopic reoperation, this patient is currently free of symptoms.

Evaluation of Symptoms

The predominant symptoms before surgery, as graded by the patients and shown in Table 4, improved significantly ($P < 0.05$) after surgery in both groups. Before surgery, 95% of the patients in both groups had no subjective swallowing difficulties.

In group 1, 16 patients (4.4%) suffered from mild to moderate dysphagia; two patients (0.5%) had had severe dysphagia preoperatively. Five patients (2.9%) in group 2 had mild to moderate dysphagia, and two patients (1.2%) had had severe dysphagia before surgery. In the early postoperative period (1 week), nearly 50% of patients in both groups had mild to moderate swallowing difficulties. In group 2, severe dysphagia developed in four patients (2.3%) within the first 8 weeks postoperatively but resolved spontaneously without any further treatment.

Three months postoperatively, a significant difference ($P < 0.05$) in subjective grading of dysphagia could be evaluated. In group 1, almost 80% of the patients were free of any swallowing difficulties. Sixty-seven patients (18.7%) had mild to moderate dysphagia, and four patients had severe dysphagia (1.1%). In group 2, 59 patients (35.3%) had swallowing difficulties, which were mild to moderate in 57 patients (34.1%) and severe in two patients (1.2%).

One year after surgery, 95% of the patients in both groups are free of any dysphagia. In group 1, 17 patients (4.9%) had mild dysphagia for solid food and none for liquids; in group 2, seven patients (4.4%) had mild dysphagia.

Table 4. Percentage of GERD symptoms before and after laparoscopic antireflux surgery in both groups

	GERD symptoms								
	Before surgery			3 mo postoperatively			1 yr postoperatively		
	None	Mild to moderate	Severe	None	Mild to moderate	Severe	None	Mild to moderate	Severe
Heartburn									
Group 1	14.1%	11.7%	74.2%	99.2%	0.8%	0%	99.4%	0.6%	0%
Group 2	12.9%	11.2%	75.9%	98.8%	1.2%	0%	99.4%	0.6%	0%
Regurgitation									
Group 1	33.0%	23.2%	43.8%	98.6%	0.8%	0.6%	99.7%	0.3%	0%
Group 2	32.4%	20.0%	47.6%	98.2%	1.2%	0.6%	100%	0%	0%
Chest pain									
Group 1	47.1%	21.3%	31.6%	88.8%	9.5%	1.7%	89.4%	10.6%	0%
Group 2	45.9%	19.4%	34.7%	89.2%	8.4%	2.4%	88.0%	11.4%	0.6%
Dysphagia									
Group 1	95.0%	4.4%	0.5%	80.2%	18.7%	1.1%	95.1%	4.9%	0%
Group 2	95.9%	2.9%	1.2%	64.7%	34.1%	1.2%	95.6%	4.4%	0%

DISCUSSION

LARS has been shown to be an established long-term treatment for chronic GERD.[3] A review of results achieved with open Nissen fundoplication shows that the procedure will result in long-term control of reflux symptoms in more than 90% of patients.[10] During the past few years, at some centers laparoscopic Nissen fundoplication has become the standard procedure for treating GERD. Excellent clinical outcomes have been achieved with LARS, with success rates ranging from 85% to 95% and with lower morbidity and mortality compared to the open approach.[11–14]

Despite these excellent results, there have been some reports of an increasing incidence of complications after LARS.[15–21] Failure of antireflux surgery, regardless of whether it was performed as an open procedure or laparoscopically, can result in the persistence of GERD symptoms and also in the development of new symptoms such as dysphagia, gas bloat, or diarrhea. One of the most frequently occurring anatomic failure after laparoscopic fundoplication is migration of the wrap into the chest,[22,23] with or without disruption of the wrap.[24,25] This so-called slipped Nissen fundoplication may be the result of inadequate closure of the diaphragmatic crura or rupture of the sutures placed at the crura. Other factors such as a short esophagus or inadequate mobilization of the esophagus leading to increasing tension on the wrap[6] can lead to a slipped fundoplication too. A slipped Nissen is more likely to occur after the laparoscopic procedure.[18] This may be due to the tendency to extend the dissection of the esophagus further into the thorax.[7] Resulting symptoms of a slipped Nissen can be dysphagia, recurrent reflux, or a combination of the two.

In a prospective study published by Soper and Dunnegan,[6] symptomatic anatomic fundoplication failure developed in 20 (7%) of 290 patients. In 13 of these patients, intrathoracic migration of the wrap with subsequent pain or GERD symptoms was the reason for failure. Horgan et al.[18] reported approximately 48 patients who had previously undergone open or laparoscopic antireflux procedures. Paraesophageal herniation of the wrap has been found to be the most frequently occurring complication after primary laparoscopic fundoplication. In addition to a thorough preoperative evaluation with a correct diagnosis and an experienced surgeon performing the optimal surgical procedure, it has been suggested that some of the technical aspects of LARS help prevent postoperative complications. Routine hiatal closure will reduce the incidence of postoperative paraesophageal hiatal hernia and a slipped Nissen. Therefore there is general agreement among surgeons performing LARS that hiatal closure should be performed routinely, regardless of whether or not a hiatal hernia is present.[6,19,26]

After more than 500 laparoscopic antireflux procedures with routine hiatal closure in our surgical unit, postoperative herniation of the Nissen valve into the chest has been shown to be the most common reason for failure. In the initial series of 361 patients, we observed intrathoracic herniation of the wrap in 22 patients (6.1%). This complication is not an automatic indication for redo surgery. Only 17 patients had symptoms such as recurrent reflux or dysphagia; five patients with Nissen valve herniation

were free of symptoms postoperatively for up to 1 year after surgery. An analysis based on recurrent wrap herniation in relation to our institutional learning curve could not be verified. Therefore it must be stated that this complication did occur sporadically throughout our entire patient population.

The relatively high frequency of this complication has led us to the use of a 1 × 3 cm polypropylene mesh for hiatal closure since December 1998 in all patients who have undergone LARS.[27] Some investigators have suggested that use of mesh prostheses in hiatal repair has proved to be a protective factor with regard to recurrent paraesophageal hiatal hernia or "slipped Nissen."

Prosthetic reinforcement for hiatal hernia repair has been used successfully by Carlson et al.[26] In a randomized trial of 31 patients, laparoscopic primary repair of large hiatal hernias was compared with laparoscopic primary repair reinforced with a polytetrafluoroethylene (PTFE) mesh. After a follow-up of 12 to 36 months, they observed a significantly lower rate of recurrent herniation in the group with PTFE reinforcement compared to the group with primary repair alone. Paul et al.[21] reported approximately three patients who underwent laparoscopic repair of large diaphragmatic defects using an expanded PTFE patch secured with intracorporal suturing techniques. All patients underwent successful repair with excellent long-term results. In a study reported by Basso et al.[28], a 3 × 4 cm polypropylene mesh was used for hiatal closure in 67 patients. After a mean follow-up period of 22.5 months, Basso et al. found no evidence of postoperative paraesophageal hernia or other mesh-related complications in comparison to 65 patients with simple sutures for hiatal closure, with an occurrence of postoperative Nissen valve herniation in 13.8% of patients. It has been shown that the use of a prolene mesh for hiatal closure is safe.[23,29,30] The weak muscular structures of the crura will be strengthened by the scar tissue that develops when a mesh prosthesis is used. The mesh is well incorporated and allows rapid mobilization of the patient. It might be a drawback, however, in terms of the possibility for erosion of a foreign body into the esophagus or transmural migration of surgical material into the esophagus after Nissen fundoplication.[31] In fact, the mesh itself does not come in contact with the posterior portion of the esophagus, but rather with the posterior part of the fundic wrap. It has been reported that there is a possibility that the fundic wrap will adhere to the mesh, which anchors the wrap in its normal anatomic position and prevents migration by itself.[17] Based on our experiences, we believe that erosion of a foreign body or migration of the mesh into an organ is a very rare occurrence and one that has not been described until now after laparoscopic placement in the hiatal region.

In the present study we compared the outcomes of 361 patients who had simple polypropylene sutures to close the hiatus with those of 170 patients who underwent routine mesh-hiatoplasty for hiatal closure. In the second series of 170 patients, only one patient (0.6%) had postoperative herniation of the wrap into the chest within the first year postoperatively.

After a follow-up period up to 1 year, no significant differences in functional outcome parameters such as esophageal manometry or 24-hour pH monitoring could be found. Comparison of the two groups in this series shows a significant change in the surgical outcome according to postoperative Nissen valve herniation. Since we began using polyvinyl mesh hiatoplasty, we have seen a decrease in postoperative wrap herniation to 0.6% (see Table 3).

One of the main problems with antireflux surgery seems to be the postoperative dysphagia. The incidence of dysphagia after Nissen fundoplication is reported to range from 3% to 24% in the literature. There is a significant correlation between the development of postoperative dysphagia and the type of operation being performed.[20] Dysphagia as a common complication after Nissen fundoplication is usually mild and improves within the first 3 months postoperatively. In our patients there was a significantly higher rate of postoperative dysphagia in group 2 with additional mesh-hiatoplasty within the first 3 months postoperatively. One year after surgery, a comparison of the two surgical groups no longer shows a significant difference in the extent of postoperative dysphagia. In general, besides several physiologic explanations for dysphagia[32] psychological factors also play a major role in the possibility of normal swallowing after LARS.[33] We believe that hiatal closure with a mesh not only reinforces parts of the natural antireflux barrier, but it also increases the time needed for adaptation of postoperative eating behavior, which can be influenced by additional psychological intervention.[34]

Our first experiences with a polypropylene-mesh for hiatal closure has been shown to be effective in preventing a slipped Nissen. It can be performed safely with no deaths and a low rate of postoperative morbidity. Despite increased postoperative dysphagia for the first 3 months after surgery, mesh-hiatoplasty in LARS has a good outcome up to 1 year after surgery.

REFERENCES

1. Arnaud JP, Pessaux P, Ghavami B, Flament JB, Trebuchet G, Meyer C, Hutten N, Champault G. Laparoscopic fun-

doplication for gastroesophageal reflux. Multicenter study of 1470 cases. Chirurgie 1999;124:516–522.
2. Cushieri A, Hunter J, Wolfe B, Swanstrom LL, Hutson W. Multicentric prospective evaluation of laparoscopic antireflux surgery: Prelminary report. Surg Endosc 1993;7:505–510.
3. Hinder RA, Filipi CJ, Wetscher G, Neary P, DeMeester TR, Perdikis G. Laparoscopic Nissen fundoplication is an effective treatment for gastroesophageal reflux disease. Ann Surg 1994;220:472–483.
4. Kuster GG, Gilroy S. Laparoscopic technique for repair of paraesophageal hiatal hernias. J Laparoendosc Surg 1993; 3:331–338.
5. Rieger NA, Jamieson GG, Britten-Jones R, Tew S. Reoperation after failed antireflux surgery. Br J Surg 1994;81: 1159–1161.
6. Soper NJ, Dunnegan D. Anatomic fundoplication failure after laparoscopic antireflux surgery. Ann Surg 1999; 229:669–677.
7. Swanstrom LL, Pennings JL. Safe laparoscopic dissection of the gastroesophageal junction. Am J Surg 1995;169:507–511.
8. Watson DI, Jamieson GG, Devitt PG, Mitchell PC, Game PA. Paraesophageal hiatus hernia: An important complication of laparoscopic Nissen fundoplication. Br J Surg 1995;82:521–523.
9. Bammer T, Kamolz T, Pasiut M, Pointner R. Minimally invasive antireflux surgery. Acta Chir Aust 2000;32:21–24.
10. DeMeester TR, Bonavina L, Albertucci M. Nissen fundoplication for gastroesophageal reflux disease. Evaluation of primary repair in 100 consecutive patients. Ann Surg 1986;204:9–20.
11. Frantzides CT, Richards C. A study of 362 consecutive laparoscopic Nissen fundoplications. Surgery 1998;124:651–654.
12. Fuchs KH, Feussner H, Bonavina L, Collard JM, Coosemans W. Current status and trends in laparoscopic antireflux surgery: Results of a consensus meeting. Endoscopy 1997;29:298.
13. Granderath FA, Kamolz T, Schweiger UM, Bammer T, Pointner R. Quality of life, surgical outcome and patients' satisfaction three years after laparoscopic antireflux surgery [abstr]. Gastroenterology 2001;120:A477.
14. Kamolz T, Wykypiel H Jr, Bammer T, Pointner R. Lebensqualität nach laparoskopischer Antirefluxchirurgie—Nissen-Fundoplikatio. Chirurg 1998;69:947.
15. Behrns KE, Schlinkert RT. Laparoscopic management of paraesophageal hernia: Early results. J Laparoendosc Surg 1996;6:311–317.
16. Cushier A, Shimi S, Nathanson LK. Laparoscopic reduction, crural repair and fundoplication of large hiatal hernia. Am J Surg 1992;163:425–430.
17. Edelman DS. Laparoscopic paraesophageal hernia repair with mesh. Surg Laparosc Endosc 1995;5:32–37.
18. Horgan S, Pohl D, Bogetti D, Eubanks T, Pellegrini C. Failed antireflux surgery. What have we learned from reoperations? Arch Surg 1999;134:809–817.
19. Hunter JG, Smith CD, Branum GD, Waring JP, Trus TL, Cornwall M, Galloway K. Laparoscopic fundoplication failures: Patterns of failure and response to fundoplication revision. Ann Surg 1999;230:595–604.
20. Hunter JG, Swanstrom L, Waring JP. Dysphagia after laparoscopic antireflux surgery. The impact of operative technique. Ann Surg 1995;224:51–57.
21. Paul MG, DeRosa RP, Petrucci PE, Palmer ML, Danovitch SH. Laparoscopic tension-free repair of large paraesophageal hernias. Surg Endosc 1997;11:303–307.
22. Huntington TR. Laparoscopic mesh repair of the esophageal hiatus. J Am Coll Surg 1997;184:399–400.
23. Kuster GG, Innocenti FA. Laparaoscopic anatomy of the region of the esophageal hiatus. Surg Endosc 1997;11:883–893.
24. Cadiere GB, Bruyns J, Himpens J, Vertruyen M. Intrathoracic migration of the wrap after laparoscopic Nissen fundoplication. Surg Endosc 1996;10:187.
25. Pointner R, Bammer T, Then P, Kamolz T. Laparoscopic refundoplication after failed antireflux surgery. Am J Surg 1999;178:541–543.
26. Carlson MA, Richards CG, Frantzides CT. Laparoscopic prosthetic reinforcement of hiatal herniorrhaphy. Dig Surg 1999;16:407–410.
27. Granderath FA, Schweiger UM, Kamolz T, Bammer T, Pasiut M, Haas CF, Pointner R. Laparoscopic antireflux surgery with routine mesh-hiatoplasty in the treatment of gastroesophageal reflux disease [abstr]. Gastroenterology 2001;120:A480.
28. Basso N, De Leo A, Genco A, Rosato P, Rea S, Spaziani E, Primavera A. 360° laparoscopic fundoplication with tension—free hiatoplasty in the treatment of symptomatic gastroesophageal reflux disease. Surg Endosc 2000;14:164–169.
29. Medina L, Peetz M, Ratzer E, Fenoglio M. Laparoscopic paraesophageal hernia repair. J Soc Laparoendosc Surg 1998;2:269–272.
30. Pitcher DE, Curet MJ, Martin DT, Vogt DM, Mason J, Zucker KA. Successful laparoscopic repair of paraesophageal hernia. Arch Surg 1997;130:590–596.
31. Arendt T, Stuber E, Monig H, Folsch UR, Katsoulis S. Dysphagia due to transmural migration of surgical material into the esophagus nine years after Nissen fundoplication. Gastrointest Endosc 2000;51:607–610.
32. Wills VL, Hunt DR. Dysphagia after antireflux surgery. Br J Surg 2001;88:486–499.
33. Kamolz T, Bammer T, Pointner R. Predictability of dysphagia after laparoscopic Nissen fundoplication. Am J Gastroenterol 2000;95:408–414.
34. Kamolz T, Granderath FA, Bammer T, Pasiut M, Pointner R. Psychological inervention influences the outcome of laparoscopic antireflux surgery in patients with stress-related symptoms of gastroesophageal reflux disease. Scand J Gastroenterol 2001;36:800–805.

Safety and Efficacy of Postoperative Continuous Positive Airway Pressure to Prevent Pulmonary Complications After Roux-en-Y Gastric Bypass

Sergio Huerta, M.D., Scott DeShields, M.S., Robert Shpiner, M.D., Zhaoping Li, M.D., Carson Liu, M.D., Mark Sawicki, M.D., James Arteaga, M.D., Edward H. Livingston, M.D.

Continuous positive airway pressure (CPAP) is used to prevent apneic arrest and/or hypoxia in patients suffering from obstructive sleep apnea. This modality has not been universally accepted for patients following upper gastrointestinal surgery because of concerns that pressurized air will inflate the stomach and proximal intestine, resulting in anastomotic disruption. This study was performed to assess the safety and efficacy of postoperative CPAP for patients undergoing a gastrojejunostomy as part of a Roux-en-Y gastric bypass (RYGB) procedure. A total of 1067 patients (837 women [78%] and 230 men [22%]) were prospectively evaluated for the risk of developing anastomotic leaks and pulmonary complications after the RYGB procedure. Of the 1067 patients undergoing gastric bypass, 420 had obstructive sleep apnea and 159 were dependent on CPAP. There were 15 major anastomotic leaks, two of which occurred in CPAP-treated patients. Contingency table analysis demonstrated that there was no correlation between CPAP utilization and the incidence of major anastomotic leakage ($P = 0.6$). Notably, no episodes of pneumonia were diagnosed in either group. Despite the theoretical risk of anastomotic injury from pressurized air delivered by CPAP, no anastomotic leaks occurred that were attributable to CPAP. There were no pulmonary complications in a patient population that is at risk for developing them postoperatively. CPAP is a useful modality for treating hypoventilation after RYGB without increasing the risk of developing postoperative anastomotic leaks. (J GASTROINTEST SURG 2002;6:354–358.)

KEY WORDS: Obstructive sleep apnea, anastomosis, pneumonia, anastomotic dehiscence

The incidence of obesity is increasing in epidemic proportions in the United States. Overweight patients are at increased risk for the development of comorbid conditions such as diabetes, hypertension osteoarthritis, hypercholesterolemia, and obstructive sleep apnea (OSA). Four out of five obese persons have at least one debilitating illness associated with this disease,[1] resulting in an increased risk of death, twofold greater in women and 12-fold greater in men.[2] Because of their medical comorbid conditions, obese patients pose a higher operative risk than nonobese individuals. OSA, defined as transient respiratory cessations during sleep, occurs frequently in obese patients. Obese patients have a 12- to 30-fold increased risk for the development of OSA relative to the general population.[3] Typically associated with upper body obesity,[4] OSA is found in 50% of obese men and 40% of obese women.[5] Frequent apneic events associated with hypoxemia severely compromise the cardiorespiratory system.[6,7]

The presence of OSA poses an increased risk for respiratory complications in obese patients undergoing laparotomy. Abdominal surgery, especially upper abdominal surgery, adversely affects pulmonary function.[8] The combination of preexisting OSA and laparotomy significantly increases morbidity and mortality from respiratory complications in obese patients.[9]

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (poster presentation).

From the Center for Human Nutrition (S.H., S.D., Z.L., J.A.) and the Department of Pulmonary Medicine (R.S.), UCLA; and Veteran Affairs of Greater Los Angeles (Z.L., C.L., M.S., E.H.L.), Los Angeles, California.

Reprint requests: Sergio Huerta, M.D., UCLA Center for Human Nutrition, 900 Veterans Ave., 12-217 Warren Hall, Box 951742, Los Angeles, CA 90095-1742. e-mail: shuerta@pol.net

Published by Elsevier Science Inc.

1091-255X/02/$—see front matter
PII: S1091-255X(01)00048-8

Continuous positive airway pressure (CPAP) effectively treats sleep apnea.[10] Continuous positive pressure is applied to the upper airway with a nasal mask, nasal prongs, or a mask that covers both the nose and the mouth.[11] CPAP improves respiratory function in morbidly obese patients[12] and accelerates reestablishment of preoperative pulmonary function[13] after upper abdominal surgery. However, the use of CPAP has not been accepted after upper intestinal surgery involving bowel anastomosis. Concerns that pressurized air will inflate the stomach and intestine resulting in anastomotic disruption have precluded the application of CPAP for treatment of postoperative apnea.

Roux-en-Y gastric bypass (RYGB) is commonly performed for the treatment of clinically severe obesity.[14–16] RYGB consists of two anastomoses: a proximal gastrojejunostomy and a distal jejunojejunostomy (Fig. 1). Both anastomoses are at risk for disruption from intraluminal distention that might result from CPAP utilization. However, CPAP decreases pulmonary complications by preventing alveolar collapse in the postoperative period after RYGB. This study was undertaken to assess the safety and efficacy of postoperative CPAP for patients having a gastrojejunostomy performed as part of a gastric bypass procedure. We specifically examined the incidence of anastomotic disruption and respiratory complications in a population of obese patients undergoing RYGB.

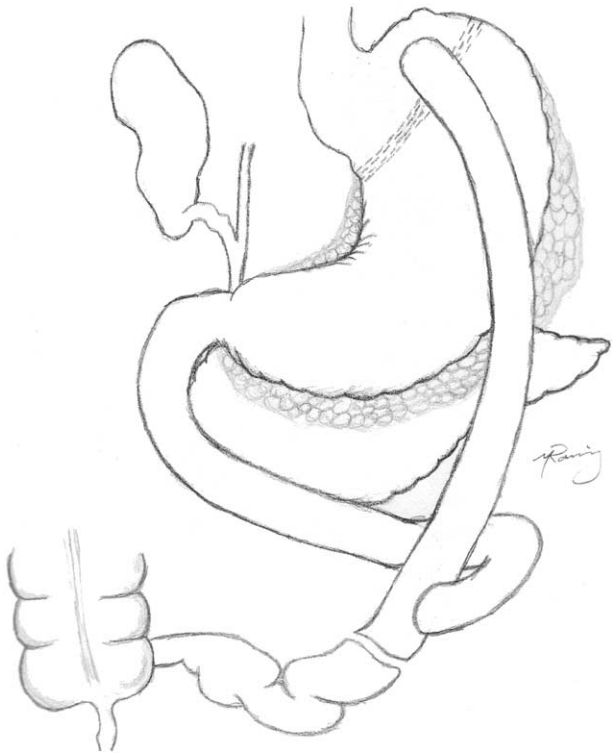


Fig. 1. The Roux-en-Y gastric bypass consists of two anastomoses. A proximal gastrojejunostomy connects the gastric pouch to the jejunal limb. The Y is created by a jejunojejunostomy approximately 50 cm distal to the gastrojejunostomy.

METHODS

Patients

All patients undergoing gastric bypass surgery at the UCLA Medical Center between December 1993 and June 2000 were included in the study. Clinical information regarding sleep apnea and the need for CPAP was entered into a database. A total of 1067 patients (837 women [78%] and 230 men [22%]) were prospectively evaluated for the risk of developing anastomotic leakage with and without CPAP. The hospital quality assurance coordinator monitored outcomes for all patients.

Surgery

All RYGB operations were performed by one of four bariatric surgeons using a standardized technique. Briefly, the proximal 30 ml gastric pouch was created by firing an Ethicon TLH-60 heavy wire stapler (Ethicon, Somerville, New Jersey) horizontally across the stomach. The jejunum was divided 30 cm distal to the ligament of Treitz and the first arcade of mesenteric vessels divided with a vascular GIA stapler (Ethicon). The distal cut end of the jejunum was then tunneled through the transverse mesocolon to lie anterior to the stomach. All anastomoses were hand sewn. The gastrojejunostomy was created by sewing the limb to the pouch side to side with a single layer of 3-0 Maxon sutures (Ethicon) over a 32 F bougie catheter, creating a 1 cm anastomosis. The small bowel anastomosis was performed side to side in two layers using an inner layer of running 3-0 Maxon sutures and an outer layer of interrupted 3-0 silk sutures. This jejunojenuostomy was created 40 to 50 cm distal to gastrojejunostomy. No attempt was made to test the anastomosis by injection of air or dye. The abdomen was closed with either interrupted No.1 Maxon sutures or a running 0 looped Maxon suture. The skin was closed with a running, continuous 4-0 Monocryl suture or skin staples (Ethicon). Postoperative nasogastric decompression was not routinely used.

Outcomes

All gastric bypass operations at the UCLA Medical Center were considered index procedures during the study period. A hospital-based quality assurance coordinator collected outcomes data independent of

the surgical team. Data concerning length of hospital stay and complications were entered into a database. All complications such as the development of pneumonia or leaks were reviewed. In addition to the independent review by the quality assurance coordinator, the attending physicians and/or house staff reported complications to the quality assurance manager to ensure that all complications were captured.

Statistical Analysis

Effects of CPAP and anastomotic leakage were analyzed by contingency table analysis. Statistical significance relating risk factors with continuous variables to the outcomes was determined by *t* test or analysis of variance where appropriate. Risk factors with dichotomous variables were tested by chi-square analysis. All data are presented as means ± standard error of the mean. All values were considered statistically significant at $P \leq 0.05$.

RESULTS

Patient Demographics

During the study period, 1067 patients underwent RYGB operations. Table 1 summarizes the patient demographic information. Seventy-eight percent of the patients were women. The male patients were taller and larger than the female patients. Comorbid conditions were more frequent in the men ($P \leq 0.05$). Of the 1067 patients undergoing gastric bypass, 420 had obstructive sleep apnea. Use of CPAP was significantly more frequent in men compared to women ($P \leq 0.05$). Of the 429 patients with sleep apnea, 159 had sleep studies documenting the need for and efficacy of CPAP for the avoidance of nocturnal apneic events. Before surgery, this patient cohort was receiving home CPAP. All of these patients received postoperative CPAP using their preoperative CPAP settings (range 10 to 12 cm H_2O at a rate of 12 to 16 cycles/min).

Table 1. Patient demographics

	Total population	Female	Male
Number	1067	837	230
Age (yr)	42.3 ± 0.3	42.2 ± 0.3	42.4 ± 0.7
Weight (pounds)	334 ± 2	313 ± 2	408 ± 6*
Height (inches)	66.1 ± 0.1	64.9 ± 0.1	70.6 ± 0.2*
Body mass index (Kg/m^2)	53.6 ± 0.3	52.4 ± 0.3	57.9 ± 0.8†
Smoking history	171 (16%)	130 (16%)	41 (18%)
Sleep apnea	420 (39%)	287 (34%)	133 (58%)†
CPAP dependency	159 (15%)	102 (12%)	57 (25%)†

Data are presented as mean ± standard error of the mean. Percentages in parentheses are relative to the number of patients referred to in a column. In our population there were 22% males and 78% females.

*$P < 0.05$ females vs. males, *t* test.

†$P < 0.05$ females vs. males, chi-square analysis.

Anastomotic Leakage

There were a total of 15 major anastomotic leaks (Table 2). The body mass index of patients with leaks is comparable to that in patients without anastomotic disruption: (52 ± 1.7 vs. 53.6 ± 0.3; $P = 0.24$, respectively). On average, the patients who developed leaks were slightly older than the general bariatric population (46 ± 2.1 years vs. 42.3 ± 0.3 years; $P \leq 0.001$, respectively).

All anastomotic leaks were confirmed radiographically by Gastrografin upper gastrointestinal series or by CT studies (Table 3). Only two of the leaks occurred in patients while they were receiving CPAP. The management strategy for treating anastomotic disruption is presented in Table 3. Contingency table analysis revealed that CPAP was not causally related to the development of postoperative anastomotic disruption after RYGB ($P = 0.65$; Table 4).

Respiratory Complications

There were no respiratory complications such as pneumonia or episodes of apnea in any patients after RYGB in our series.

DISCUSSION

The incidence of obesity is increasing in the United States, resulting in the performance of a greater number of bariatric operations. RYGB has become the "gold standard" operation for the treatment of clinically severe obesity.[15,16] Obese patients are at risk for postoperative complications after RYGB because of their greater preoperative disease burden. Despite the high surgical risk, the overall complication rate remains low. However, complications do occur and analysis of predictive risk factors is important to minimize the rate of complications.

Because of the high incidence of OSA in obese patients, these patients have a uniquely high risk for developing postoperative apnea, hypoxia, and pneumonia. This condition is effectively treated by CPAP. However, because of the concern that positive airway pressure places gastrointestinal anastomoses at risk for disruption, CPAP has not been routinely used in postoperative patients. The current study, which pro-

Table 2. Characteristics of patients who had leaks postoperatively vs. the total population

	Patients with leaks (N = 15)	Total population (N = 1067)
Age (yr)	46 ± 2.1	42.3 ± 0.3
Body mass index	52 ± 1.7	53.0 ± 0.3
Females	8 (53%)	837 (78%)
Males	7 (47%)	230 (22%)

spectively evaluated a consecutive series of 1067 gastric bypass operations, has demonstrated the safety and efficacy of CPAP utilization after RYGB.

The incidence of OSA among morbidly obese patients is very high.[4] In our series, 39% of patients presented with OSA, of whom 38% were CPAP dependent and were using this modality at home for the management of their OSA. Obstructive sleep apnea is a common risk factor for the development of respiratory complications following RYGB.[15–17] Aside from OSA, obese patients have compromised respiratory function characterized by decreased functional residual capacity, expiratory reserve volume, PaO_2, and an increase in the alveolar-arterial oxygen difference.[18] Taken together, these factors result in an increased risk for postoperative pulmonary complications such as sputum retention, atelectasis, and bronchopulmonary infections,[8] which cause significant postoperative morbidity and mortality after elective surgery. Gastric bypass series similar to the one presented here report rates of atelectasis and pneumonia ranging from 0.5% to 4%.[15–17]

Continuous airway pressure is currently the most effective medical treatment for OSA.[4] CPAP restores functional residual capacity to preoperative values[19,20] and improves oxygenation[21] after surgery. The prophylactic use of airway pressure systems during the first 24 hours postoperatively significantly reduces the pulmonary restrictive syndrome that occurs after gastroplasty in morbidly obese patients.[13] It also reduces the risk of acute respiratory distress syndrome after upper abdominal surgery.[22] The mechanical effect of CPAP is achieved by raising the intraluminal upper airway pressure. Thus the postoperative use of CPAP carries the theoretical risk of increasing the incidence of anastomotic leaks resulting from the increase in pressurized air into the stomach and proximal anastomosis. Fear of anastomotic disruption resulting from injection of air into the bowel has limited the use of CPAP in postoperative patients.

Although the percentage of anastomotic leaks in our series (1.4%) is slightly higher than that in other reports (0.5% to 1%),[15–17] our analysis demonstrates that the increase is not attributable to the use of CPAP. We routinely use CPAP for patients with sleep apnea. We also use it for patients with postoperative respiratory insufficiency. Despite concerns that positive pressure ventilation will result in anastomotic disruption, we did not identify a relationship between the use of CPAP and anastomotic disruption. Our findings and conclusions are limited to the population we studied: obese patients undergoing RYGB. Malnourished patients or those who are undergoing surgery for a malignancy may not have the same outcome because of compromised wound healing, which is not characteristic of RYGB patients.

We conclude that CPAP is a useful modality for treating patients at risk for apnea after RYGB. There were no pulmonary complications in a high-risk patient population and no anatomic leaks were attributable to the use of CPAP. We recommend the routine use of postoperative CPAP for RYGB patients with CPAP-dependent OSA.

Table 3. Diagnostic studies performed and management of patients with anastomotic disruption*

Patient	Diagnostic study	Management
1	Gastrografin swallow	Nonsurgical
2	Gastrografin swallow	Surgical
3	Gastrografin swallow	Surgical
4	Gastrografin swallow	Nonsurgical
5	Gastrografin swallow	Surgical
6	Gastrografin swallow	Surgical
7	Gastrografin swallow	Surgical
8	CT-scan	Nonsurgical
9	CT-scan	Nonsurgical
10	CT-scan	Surgical
11	CT-scan	Surgical
12	CT-scan	Surgical
13	CT-scan	Surgical
14	CT-scan	Surgical
15	CT-scan	Surgical

In 47% of the patients, the diagnosis was made by Gastrografin upper gastrointestinal series. Sixty-seven percent of the patients required surgical intervention for the management of the leaks.

Table 4. Contingency table analysis for CPAP and leakage

	(−) CPAP	(+) CPAP	Total/Leak
(−) Leak	895	157	1052
(+) Leak	13	2	15
Total/CPAP	908	159	

N = 1067; $P = 0.648$.

REFERENCES

1. Mun EC, Blackburn GL, Matthews JB. Current status of medical and surgical therapy for obesity. Gastroenterology 2001;120:669–681.
2. Drenick EJ, Bale GS, Seltzer F, Johnson DG. Excessive mortality and causes of death in morbidly obese men. JAMA 1980;243:443–445.
3. Peiser J, Lavie P, Ovnat A, Charuzi I. Sleep apnea syndrome in the morbidly obese as an indication for weight reduction surgery. Ann Surg 1984;199:112–115.
4. Kyzer S, Charuzi I. Obstructive sleep apnea in the obese. World J Surg 1998;22:998–1001.
5. Richman RM, Elliott LM, Burns CM, Bearpark HM, Steinbeck KS, Caterson ID. The prevalence of obstructive sleep apnoea in an obese female population. Int J Obes Relat Metab Disord 1994;18:173–177.
6. Cheshire K, Engleman H, Deary I, Shapiro C, Douglas NJ. Factors impairing daytime performance in patients with sleep apnea/hypopnea syndrome. Arch Intern Med 1992; 152:538–541.
7. Vgontzas AN, Tan TL, Bixler EO, Martin LF, Shubert D, Kales A. Sleep apnea and sleep disruption in obese patients. Arch Intern Med 1994;154:1705–1711.
8. Clergue F, Whitelaw WA, Charles JC, Gandjbakhch I, Pansard JL, Derenne JP, Viars P. Inferences about respiratory muscle use after cardiac surgery from compartmental volume and pressure measurements. Anesthesiology 1995;82:1318–1327.
9. Rezaiguia S, Jayr C. Prevention of respiratory complications after abdominal surgery. Ann Fr Anesth Reanim 1996; 15:623–646.
10. Strollo PJ Jr, Rogers RM. Obstructive sleep apnea. N Engl J Med 1996;334:99–104.
11. Indications and standards for use of nasal continuous positive airway pressure (CPAP) in sleep apnea syndromes. Am J Respir Crit Care Med 1994;150:1738–1745.
12. Pelosi P, Ravagnan I, Giurati G, Panigada M, Bottino N, Tredici S, Eccher G, Gattinoni L. Positive end-expiratory pressure improves respiratory function in obese but not in normal subjects during anesthesia and paralysis. Anesthesiology 1999;91:1221–1231.
13. Joris JL, Sottiaux TM, Chiche JD, Desaive CJ, Lamy ML. Effect of bi-level positive airway pressure (BiPAP) nasal ventilation on the postoperative pulmonary restrictive syndrome in obese patients undergoing gastroplasty. Chest 1997;111:665–670.
14. Howard L, Malone M, Michalek A, Carter J, Alger S, Van Woert J. Gastric bypass and vertical banded gastroplasty—A prospective randomized comparison and 5-year follow-up. Obes Surg 1995;5:55–60.
15. Sugerman HJ, Starkey JV, Birkenhauer R. A randomized prospective trial of gastric bypass versus vertical banded gastroplasty for morbid obesity and their effects on sweets versus non-sweets eaters. Ann Surg 1987;205:613–624.
16. Sugerman HJ, Londrey GL, Kellum JM, Wolf L, Liszka T, Engle KM, Birkenhauer R, Starkey JV. Weight loss with vertical banded gastroplasty and Roux-Y gastric bypass for morbid obesity with selective versus random assignment. Am J Surg 1989;157:93–102.
17. Hall JC, Watts JM, O'Brien PE, Dunstan RE, Walsh JF, Slavotinek AH, Elmslie RG. Gastric surgery for morbid obesity. The Adelaide Study. Ann Surg 1990;211:419–427.
18. Jenkins SC, Moxham J. The effects of mild obesity on lung function. Respir Med 1991;85:309–311.
19. Lindner KH, Lotz P, Ahnefeld FW. Continuous positive airway pressure effect on functional residual capacity, vital capacity and its subdivisions. Chest 1987;92:66–70.
20. Stock MC, Downs JB, Corkran ML. Pulmonary function before and after prolonged continuous positive airway pressure by mask. Crit Care Med 1984;12:973–974.
21. Pinilla JC, Oleniuk FH, Tan L, Rebeyka I, Tanna N, Wilkinson A, Bharadwaj B. Use of a nasal continuous positive airway pressure mask in the treatment of postoperative atelectasis in aortocoronary bypass surgery. Crit Care Med 1990;18:836–840.
22. Schmidt GB, Bombeck CT, Bennett EJ, Kotb KM. Continuous positive airway pressure in the prophylaxis of the adult respiratory distress syndrome (ARDS). Langenbecks Arch Chir 1975;(Suppl):439–442.

Glutathione S–Transferase-Pi Expression Is Downregulated in Patients With Barrett's Esophagus and Esophageal Adenocarcinoma

Jan Brabender, M.D., Reginald V. Lord, M.B.B.S., Kumari Wickramasinghe, M.D., Ralf Metzger, M.D., Paul M. Schneider, M.D., Ji-Min Park, M.S., Arnulf H. Hölscher, M.D., Tom R. DeMeester, M.D., Kathleen D. Danenberg, B.S., Peter V. Danenberg, Ph.D.

The glutathione S–transferases (GSTs) are a family of enzymes that play an important role in the prevention of cancer by detoxifying numerous potentially carcinogenic compounds. GSTs conjugate reduced glutathione to a variety of electrophilic and hydrophobic compounds, converting them into more soluble, more easily excretable compounds. Decreased glutathione S–transferase-pi (GSTPI) enzyme activity has been reported in Barrett's esophagus, and an inverse correlation was demonstrated between GST enzyme activity and tumor incidence in the gastrointestinal tract, but the role of GSTPI messengerRNA (mRNA) expression in Barrett's esophagus and associated adenocarcinomas is uncertain. The purpose of this study was to investigate the role of GSTPI mRNA and protein expression in the development and progression of the Barrett's metaplasia-dysplasia-adenocarcinoma sequence, and to investigate the potential of GSTPI quantitation as a biomarker in the clinical management of this disease. GSTPI mRNA expression levels, in relation to the housekeeping gene β-actin, were analyzed using a quantitative real-time reverse transcription–polymerase chain reaction method (TaqMan) in 111 specimens from 19 patients with Barrett's esophagus without carcinoma (BE group), 21 patients with Barrett's-associated adenocarcinoma (EA group), and a control group of 10 patients without evidence of Barrett's esophagus or chronic gastroesophageal reflux disease. GSTPI mRNA expression was detectable in all 111 samples investigated. Analyzed according to histopathologic group, the median GSTPI mRNA expression was highest in normal squamous esophagus epithelium, intermediate in Barrett's esophagus, and lowest in adenocarcinoma tissues ($P < 0.001$). The median GSTPI expression was significantly decreased in Barrett's esophagus tissues compared to matching normal squamous esophagus from either the BE group ($P = 0.001$) or the EA group ($P = 0.023$). GSTPI expression levels in adenocarcinoma tissues were decreased compared to matching normal esophagus tissues from the patients with adenocarcinoma ($P = 0.011$). Furthermore, GSTPI mRNA expression values were significantly different between metaplastic, dysplastic, and adenocarcinoma tissues ($P = 0.026$). GSTPI expression levels were also significantly lower in histologically normal squamous esophagus tissues from patients with cancer (EA group) compared to both normal esophagus tissues from patients without cancer (BE group; $P = 0.007$) and normal esophagus tissues from the control group with no esophageal abnormality ($P = 0.002$). GSTPI protein expression was generally highest in the basal layer of normal squamous esophagus epithelium and lowest in adenocarcinoma cells, with Barrett's cells showing intermediate staining intensity. Our results show that downregulation of GSTPI expression is an early event in the development of Barrett's esophagus and esophageal adenocarcinoma. Loss of GSTPI expression may have an important role in the development and progression of this disease. (J GASTROINTEST SURG 2002;6:359–367.)

KEY WORDS: Barrett's esophagus, esophageal carcinoma, GSTPI, gene expression

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (poster presentation).

From the Departments of Biochemistry and Molecular Biology, (J.B., J.M.P., K.D.D., P.V.D.), USC Norris Comprehensive Cancer Center and the Departments of Surgery (R.V.L.,T.R.D.) and Pathology (K.W.), University of Southern California Keck School of Medicine, Los Angeles, California; and the Department of Visceral and Vascular Surgery (J.B., R.M., P.M.S., A.H.H.), University of Cologne, Cologne, Germany.

Supported by the Burda Foundation for Cancer Research, Germany (J.B.), the American Cancer Society, and the STOP Cancer Foundation (R.V.L.), and by grant RO1 CA 71716 (P.V.D.) from the National Cancer Institute.

Reprint requests: Jan Brabender, M.D., USC/Norris Comprehensive Cancer Center, NOR 5318, 1441 Eastlake Ave., Los Angeles, CA 90033. e-mail: drbrabender@cs.com

1091-255X/02/$—see front matter
PII: S1091-255X(02)00003-3

In the past three decades, the incidence of adenocarcinoma of the esophagus has increased in many Western countries at a rate that exceeds that of any other malignancy.[1–4] The main risk factor for the development of adenocarcinoma of the esophagus is the presence of Barrett's esophagus,[5] a condition in which the normal squamous lining of the esophagus is replaced with columnar epithelium as a result of long-standing gastroesophageal reflux.[6] Barrett's esophagus is a multistep process in which Barrett's intestinal metaplasia eventually progresses to low-grade dysplasia (LGD), high-grade dysplasia (HGD), and ultimately to adenocarcinoma of the esophagus.[7] Compared to the general population, patients with Barrett's esophagus have an approximately 30- to 125-fold greater risk for adenocarcinoma of the esophagus,[5] with an estimated incidence of cancer ranging from 1 in 55 to as low as 1 in 441 patient-years.[8] The identification of novel biomarkers that are reliably associated with each stage of Barrett's disease and with an increased likelihood of progression to more advanced stages may help select patients at highest risk for malignant transformation.

Human glutathione S–transferases (GSTs) are a supergene family of enzymes involved in the phase II detoxification of toxins and enzymes.[9] Based on nucleotide homology and biochemical and immunologic criteria, four GST isoforms (α, μ, π, and θ) have been identified in humans.[10] GSTs catalyze the binding of a large variety of electrophiles to the sulfhydryl group of glutathione, converting them to less harmful and more water-soluble, more easily excretable compounds.[9,11] Because most chemical carcinogens are electrophiles, GSTs play a critical role in the detoxification of carcinogens.[9,12] GSTs are widely expressed in human epithelial tissues, including the human gastrointestinal tract,[13–16] with GST-π, also written as GST-pi or GSTPI, as the predominant isoform in the normal squamous esophagus epithelium.[16] Deficiencies of GST have been reported to enhance the risk of developing gastric, colorectal, or lung cancer,[17–19] and an inverse correlation was demonstrated between GST enzyme activity and tumor incidence in the gastrointestinal tract.[15,20] Decreased GSTPI enzyme activity has been detected in Barrett's esophagus compared to normal squamous esophagus, suggesting that this alteration may contribute to an increased cancer risk in this disease.[15,20] The analysis of GSTPI mRNA expression in Barrett's esophagus and adenocarcinoma of the esophagus has so far been limited to semiquantitative techniques with somewhat conflicting results. Compton et al.[21] reported decreased GSTPI mRNA expression in metaplastic Barrett's and adenocarcinoma tissues compared to normal squamous esophagus tissues, whereas Ishioka et al.[22] detected elevated GSTPI mRNA expression in adenocarcinomas of the esophagus. To further elucidate the role of GSTPI mRNA expression in the development and progression of Barrett's esophagus and associated adenocarcinoma, and to investigate the potential of GSTPI quantitation in the clinical management of this disease, we performed quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR; TaqMan) expression analysis on a total of 111 specimens from patients with Barrett's esophagus or Barrett's-associated adenocarcinoma of the esophagus.

MATERIAL AND METHODS

Tissue Samples for RT-PCR

A total of 111 tissue samples obtained at endoscopy and surgery from 19 patients with Barrett's esophagus without adenocarcinoma (BE group), 21 patients with Barrett's-associated esophageal adenocarcinoma (EA group), and 10 patients with no symptomatic, endoscopic, or histopathologic evidence of Barrett's esophagus or chronic gastroesophageal reflux disease (control group [CG]) were collected and immediately frozen in liquid nitrogen. There were 32 men and 19 women whose median age was 61.8 years (range 24 to 77 years). Endoscopic biopsies were obtained according to a protocol that required biopsy at 2 cm intervals from each quadrant (anterior, posterior, right, and left lateral positions) of the visible length of Barrett's mucosa and an additional biopsy from the normal-appearing squamous mucosa of the esophagus. Biopsy specimens of the normal esophagus were taken at least 4 cm proximal to the macroscopically abnormal epithelium. Part of the specimen or an adjacent specimen was fixed in formalin and paraffin for histopathologic examination.

Specimens were classified as intestinal metaplasia if intestinal metaplasia but no dysplasia or cancer was present. Specimens were classified as dysplastic if either low-grade dysplasia (LGD) or high-grade dysplasia (HGD) was present. Dysplastic tissues were not divided into LGD or HGD groups because areas of LGD and HGD were commonly present in the same specimen. Using these criteria, the following tissues were analyzed for GSTPI mRNA expression: Barrett's intestinal metaplasia (n = 16), Barrett's dysplasia (n = 3), and matching normal squamous tissue (n = 19) in the BE group, Barrett's adenocarcinoma of the esophagus (n = 21), Barrett's intestinal metaplasia (n = 5), Barrett's dysplasia (n = 16), and matching normal squamous esophagus tissues (n = 21) in the EA group, and normal squamous esophagus tissues (n = 10) in the control group, for a total of 111 specimens.

RNA Extraction and cDNA Synthesis

Total RNA was isolated by a single-step guanidinium isothiocyanate method using the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, Inc., Piscataway, New Jersey), according to the manufacturer's instructions; cDNA specimens were prepared as previously described.[23,24]

PCR Quantitation of mRNA Expression

Quantitation of GSTPI cDNA and an internal reference cDNA (β-actin) was carried out with the use of a fluorescence detection method (ABI PRISM 7700 sequence detection system [TaqMan]; Perkin-Elmer Applied Biosystems, Foster City, California), as described.[24–27]

The PCR mixture consisted of the following: 600 nmol/L of each primer; 200 nmol/L probe; 5 U AmpliTaq Gold polymerase; 200 μmol/L each dATP, dCTP, and dGTP; 400 μmol/L dUTP; 5.5 mmol/L $MgCl_2$; and 1 × TaqMan buffer A containing a reference dye, to a final volume of 25 μl (all reagents from Perkin-Elmer Applied Biosystems). Cycling conditions were 50° C for 10 seconds, 95° C for 10 minutes, followed by 46 cycles at 95° C for 15 seconds and 60° C for 1 minute.

The primers and probes used were as follows. In each single case, the first primer is the forward primer, the second is the reversed primer, and the third is the probe: GSTPI: CCTGACCCAGTCCA ATACCAT CCT and TGTAGATGAGGGAGATGTATTTG CA; probe 6FAM (carboxyfluorescein) 5′-CTTCCCA TAGAGCCCAAGGGTGCG-3′TAMRA (N,N,N′, N′-tetramethyl-6carboxyrhodamine); β-Actin: TGAG CGCGGCTAC AGCTT and TCCTTAATGTCAC GCACGATTT; probe: 6FAM5′-ACCACCACGGC-CGAGCGG-3′TAMRA.

Immunohistochemical Analysis

Twenty-five archival formalin-fixed, paraffin-embedded blocks from 25 different patients with Barrett's esophagus or adenocarcinoma were cut into 5 μm sections, mounted onto polylysine-coated slides, dewaxed in xylene, and rehydrated in alcohol. Pretreatment by immersion in 10 mmol/L citrate buffer, pH 6.0, with microwave, pressure cooker heating was performed. The sections were peroxidase blocked using 3% hydrogen peroxide in 0.05 mol/L TRIS–hydrochloric acid buffer, incubated for 15 minutes with normal horse serum, and incubated for 1 hour at room temperature with the GSTPI primary antibody (PAb-1; Neomarkers, Inc., Fremont, California) at 1:50 dilution. Biotinylated horse antimouse secondary antibody (1:200 dilution for 40 minutes; Vector Labs, Burlingame, California), peroxidase-conjugated streptavidin complex reagent (1:100 dilution for 30 minutes; VectaStain Elite ABC Kit, Vector Labs), and 3.3′-diaminobenzidine (DAB; 10 mg in 10 ml TRIS buffer for 20 minutes) were used to visualize binding of the first antibody. A liver–bile duct section (Neomarkers, Inc.) was used for a positive control and primary antibody was omitted for a negative control.

Thirty-five different histologic areas were studied in these 25 sections: 10 areas of normal squamous esophagus epithelium, 10 areas of intestinal metaplasia, and five areas each of LGD, HGD, and adenocarcinoma. The maximal intensity of staining for GSTPI in epithelium was graded from 0 to 3+, denoting no staining or light, moderate, or intense staining. An immunohistochemical staining score was calculated for each histologic area by multiplying the staining intensity level (0 to 3) by the proportion of cells in each area staining with that intensity. The immunohistochemical staining score for an area with 100% of cells staining with 3+ intensity, for example, would be 1 × 3, equaling 3, whereas an area with 50% cells staining 2+ and 40% staining 1+ would have a score of (0.5 × 2) plus (0.4 × 1), equaling 1.4.

Statistical Analysis

TaqMan analyses yielded values that are expressed as ratios between two absolute measurements (gene of interest/internal reference gene). GSTPI expression levels in adenocarcinoma, Barrett's esophagus, and normal squamous esophagus tissues were compared using the Kruskal-Wallis test to identify significant differences in expressions among the histopathologic groups. The Kruskal-Wallis test was also used to compare the three groups of normal esophagus tissues. When the overall Kruskal-Wallis test (comparing 3 groups) was significant at the 0.05 level, pairwise comparisons were based on the Mann-Whitney test and the nominal *P* value was reported. The Wilcoxon signed-rank test was used for comparison of paired tissues. Statistical significance (with two-sided tests) was set at the 0.05 level.

RESULTS

RT-PCR Results

GSTPI mRNA expression was detectable by quantitative real-time PCR (TaqMan) in all 111 specimens (100%). Analyzed according to histopathologic group, the median GSTPI mRNA expression was highest in normal squamous esophagus tissues (median 3.51, range 0.35 to 7.07), intermediate in Barrett's esopha-

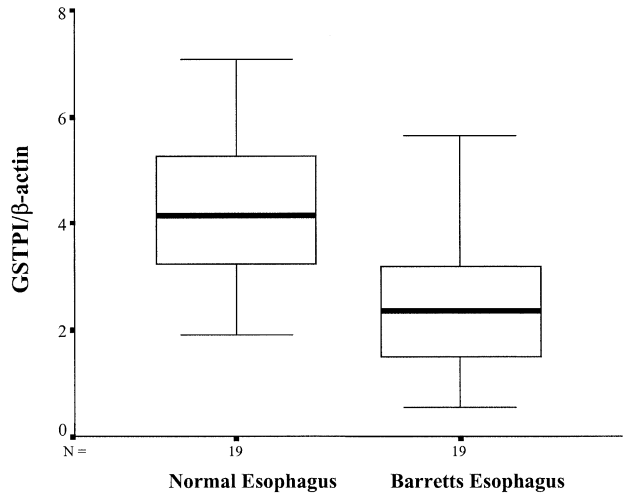


Fig. 1. Box and whisker plots of relative GSTPI mRNA expression levels for normal esophagus and Barrett's esophagus tissues from patients with the maximum diagnosis of Barrett's esophagus. The boxes show the twenty-fifth and seventy-fifth percentile (interquartile) ranges. Median values are shown as a horizontal black bar within each box. The whiskers show levels outside the twenty-fifth and seventy-fifth percentiles. $P = 0.001$.

gus (median 1.97, range 0.17 to 5.64), and lowest in Barrett's-associated adenocarcinoma of the esophagus (median 1.34, range 0.19 to 4.89; $P < 0.001$, Kruskal-Wallis test).

Seventeen (89.5%) of 19 patients with the maximum diagnosis of Barrett's esophagus (BE group, n = 19) had lower GSTPI mRNA expression levels in Barrett's epithelium compared to matching normal squamous esophagus tissues. The median GSTPI mRNA expression in normal squamous esophagus tissues was 4.14 (range 1.89 to 7.07) and 2.34 in Barrett's esophagus (range 0.55 to 5.64; $P = 0.001$, Wilcoxon test; Fig. 1 and Table 1).

In the group of patients with Barrett's-associated adenocarcinoma (EA group, n = 21), 15 (71.4%) of 21 patients had lower GSTPI mRNA expression levels in cancer tissues compared to matching normal esophagus tissues. The median GSTPI mRNA expression was 2.88 (range 0.35 to 4.91) in normal esophagus, 1.77 (range 0.17 to 4.58) in Barrett's epithelium, and 1.34 (range 0.19 to 4.90) in Barrett's-associated adenocarcinoma ($P = 0.006$, Kruskal-Wallis test). Table 1 and Fig. 2 show that the median GSTPI mRNA expression was significantly lower in Barrett's epithelium and Barrett's-associated adenocarcinoma compared to matching normal esophagus tissues.

To search for further differences in the GSTPI mRNA expression between the different stages of Barrett's progressive disease, we compared the median GSTPI expression of metaplastic esophagus (intestinal metaplasia) tissues from patients with the maximum diagnosis of Barrett's esophagus (BE group) with dysplastic Barrett's and cancer tissues from patients with adenocarcinoma of the esophagus (EA group). As shown in Fig.3, the median GSTPI expression was highest in Barrett's intestinal metaplasia (n = 16; median 2.62, range 0.55 to 5.64), intermediate in Barrett's dysplasia (n = 16; median 2.01, range 0.17 to 4.58), and lowest in adenocarcinomas of the esophagus (n = 21; median 1.34, range 0.19 to 4.90; $P = 0.026$, Kruskal-Wallis test).

Overall, the three groups of normal esophagus tissue revealed substantial differences in GSTPI expression levels ($P = 0.002$; Kruskal-Wallis test). The median GSTPI mRNA expression in the group of histologically normal squamous esophagus tissues from patients with adenocarcinoma (median 2.88, range 0.35 to 4.91) was significantly lower than the median GSTPI expression found in normal squamous esophagus tissues from patients with Barrett's esophagus only (median 4.14, range 1.89 to 7.07; $P = 0.007$, Mann-Whitney test) and normal squamous esophagus tissues obtained from the control group (median 4.58, range 3.45 to 14.72; $P=0.002$, Mann-Whitney test; Fig. 4).

Table 1. GSTPI mRNA expression in tissues from patients with adenocarcinoma and Barrett's esophagus

Pathology	n	GSTPI expression		Interquartile range (25th–75th percentiles)	*P* value
		Median	Range		
EA group	21				
Adenocarcinoma		1.34	0.19–4.90	0.71–2.14	0.006
Barrett's esophagus		1.77	0.17–4.58	0.69–2.58	
Normal esophagus		2.88	0.35–4.91	1.82–4.10	
BE group	19				
Barrett's esophagus		4.14	1.89–7.07	1.29–3.19	0.001
Normal esophagus		2.34	0.55–5.64	2.95–5.30	
CG group	10				
Normal esophagus		4.58	3.45–14.72	3.85–6.70	

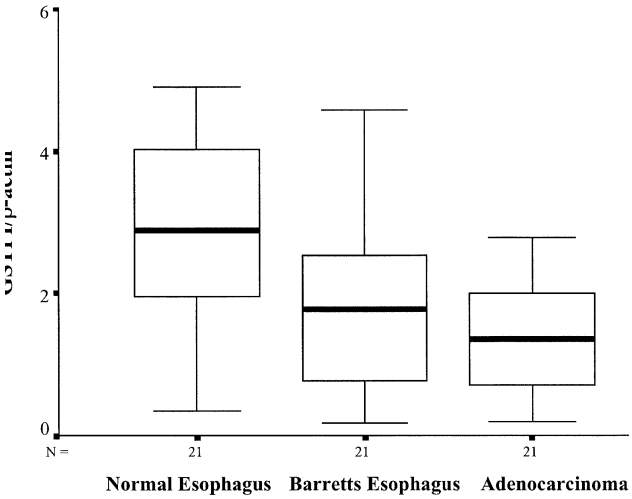


Fig. 2. Box and whisker plots of relative GSTPI mRNA expression levels for normal esophagus (NE), Barrett's esophagus (BE), and adenocarcinoma tissues from patients with Barrett's-associated adenocarcinoma of the esophagus (EA). NE vs. BE, $P = 0.023$; NE vs. EA, $P = 0.011$; BE vs. EA, P = NS.

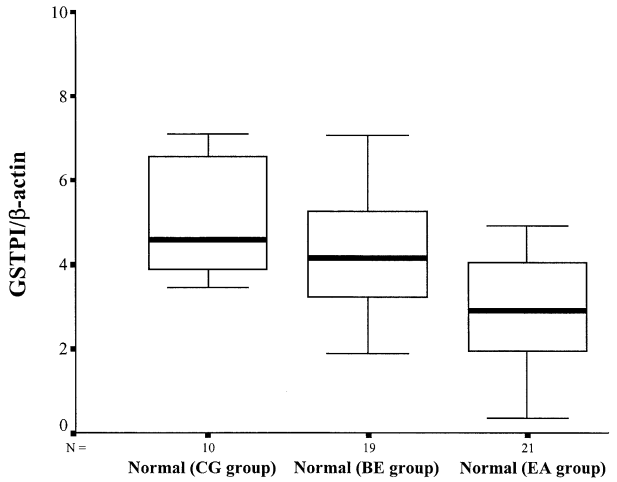


Fig. 4. Box and whisker plots of relative GSTPI mRNA expression levels for normal squamous esophagus tissues from a control group (CG) without evidence of Barrett's esophagus or chronic gastroesophageal reflux, and patients with Barrett's esophagus (BE group) and patients with adenocarcinoma of the esophagus (EA group). CG vs. BE, P = NS; CG vs. EA, $P = 0.001$; BE vs. EA, $P = 0.007$.

Immunohistochemical Results

The immunohistochemical staining score results for GSTPI nuclear staining are shown in Table 2. Nuclear staining was generally most intense in the basal layer cells in normal squamous esophagus epithelium, although there was also at least one case in the intestinal metaplasia, LGD, and HGD groups with strongly intense staining in most cells. GSTPI protein expression was similar in each of the Barrett's intestinal metaplasia, LGD, and HGD groups, but tended to be lower in adenocarcinoma cells than in either the normal squamous esophagus or the Barrett's cells (see Table 2). Cytoplasmic GSTPI staining was either faint or absent in most Barrett's cells but was present in all of the normal squamous esophagus epithelia, with moderately intense (2+) staining in most normal squamous cells. Moderately intense cytoplasmic staining was also present in two of five adenocarcinoma tissues. Representative immunohistochemically stained images are shown in Fig. 5.

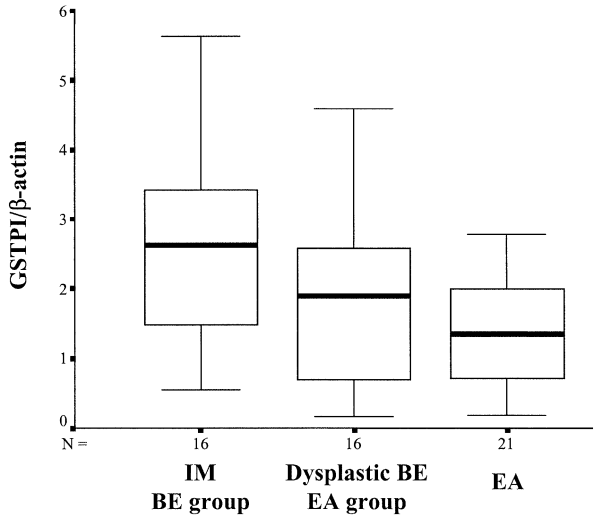


Fig. 3. Box and whisker plots of relative GSTPI mRNA expression for metaplastic esophagus tissues from patients with Barrett's esophagus, and dysplastic esophagus and adenocarcinoma tissue from patients with adenocarcinoma of the esophagus. $P = 0.026$.

Table 2. Immunohistochemical assessment of GSTPI protein expression

GSTPI nuclear staining	No. of cases	Immunohistochemical staining score*	
		Mean	Range
Normal squamous†	10	2.4	1.5–2.8
Intestinal metaplasia	10	1.1	0.3–2.2
Low grade dysplasia	5	1.1	0.9–1.5
High grade dysplasia	5	1.3	0.8–2.0
Adenocarcinoma	5	1.0	0.8–1.2

*Calculated by multiplying the staining intensity (0 to 3) by the proportion of positively staining abnormal cells (see Material and Methods for details).
†Basal layer of normal squamous esophagus epithelium.

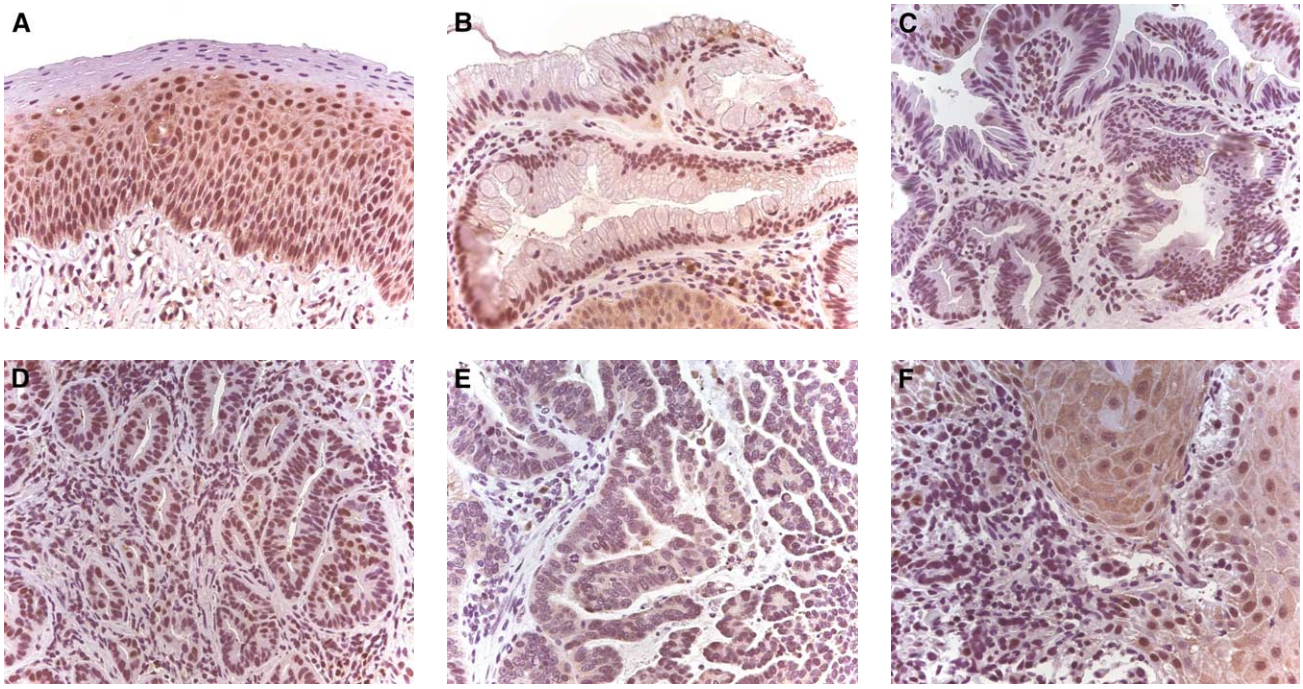


Fig. 5. Representative immunohistochemically stained images showing GSTPI protein expression in normal squamous esophagus, Barrett's esophagus, and esophageal adenocarcinoma. Uniform nuclear staining is present in the normal squamous esophagus cells, but only nonuniform nuclear staining is seen in intestinal metaplasia, LGD, HGD, and adenocarcinoma cells. (Original magnification for *A–F*, ×400.) **A,** Normal squamous esophagus showing intense brown nuclear GSTPI staining and moderate cytoplasmic staining, especially in the basal layer cells. **B,** Barrett's esophagus intestinal metaplasia showing nonuniform nuclear staining but little cytoplasmic staining. An area of normal squamous esophagus is seen at the bottom of the figure. **C,** Barrett's esophagus with low-grade dysplasia. **D,** Barrett's esophagus with high-grade dysplasia. **E,** Esophageal adenocarcinoma. **F,** Esophageal adenocarcinoma with faint or absent GSTPI protein expression adjacent to areas of normal squamous esophagus with moderate nuclear and cytoplasmic immunoreactivity.

DISCUSSION

The main risk factor for the development of esophageal adenocarcinoma is the presence of Barrett's esophagus. The mechanisms underlying the increased cancer development in this tissue are not fully understood, but substantial evidence exists that progression to Barrett's cancer is associated with a variety of genetic and epigenetic alterations.[28–31] Decreased levels of protective enzymes such as the GSTs might increase the potential for the esophageal mucosa to accumulate certain genetic or epigenetic alterations that ultimately lead to the development of cancer. Several studies have reported lower GSTPI enzyme activity and enzyme content in Barrett's esophagus and esophageal adenocarcinoma compared to normal squamous esophagus,[15,20] and Compton et al.[21] found lower GSTPI mRNA levels using Northern blot analysis in Barrett's metaplasia compared to normal squamous esophagus biopsy specimens from the same patients. The GSTPI mRNA levels were higher in adenocarcinoma than in Barrett's metaplasia in the study by Compton et al.,[21] however, which suggests that further studies on the GSTPI expression patterns in this disease are warranted.

We used a quantitative real-time RT-PCR (TaqMan) method to analyze the GSTPI mRNA expression, and immunohistochemical analysis to assess GSTPI protein expression in all histopathologic subtypes of Barrett's esophagus and Barrett's-associated adenocarcinoma of the esophagus. We found that GSTPI mRNA and protein expression is decreased in both Barrett's esophagus and cancer tissues compared with matching normal esophagus tissues. Our results suggest that downregulation of GSTPI mRNA expression is an early event in the development of Barrett's esophagus and might be associated with the progression to adenocarcinoma. In contrast to the study by Compton et al.,[21] mRNA expression levels were generally lower in adenocarcinoma compared to Barrett's tissues.

The mechanism for decreased GSTPI mRNA expression in this disease is not known. GSTPI mRNA expression was proportional to GSTPI enzyme ac-

tivity in Barrett's esphagus in the study by Compton et al.,[21] suggesting that expression is controlled at the transcriptional level. Van Lieshout et al.[32] reported an inverse association between the occurrence of the GSTPIb polymorphism and GSTPI enzyme activity in patients with Barrett's esophagus, indicating that variations in the DNA sequence may be a mechanism for downregulating GSTPI mRNA expression. Another possible mechanism for transcriptional silencing is hypermethylation of CpG islands within promoter and 5′ regions of various genes.[33,34] Hypermethylation of the GSTPI gene in adenocarcinoma of the esophagus has been reported recently, although at a low frequency.[31] Further studies are warranted to determine the underlying mechanisms leading to decreased GSTPI mRNA expression in this disease.

We did not find any marked differences in GSTPI protein expression in tissues from the Barrett's stages of intestinal metaplasia, LGD, and HGD. Similarly, van Lieshout et al.[32] reported lower GST enzyme activity and content in Barrett's esophagus compared to normal esophagus mucosa, but no differences between the different stages of Barrett's esophagus. Using the fluorescent real-time RT-PCR (TaqMan) method for GSTPI expression analysis, in contrast, enabled us to detect significantly different GSTPI mRNA levels between metaplastic and dysplastic Barrett's tissues. The differences between the protein or enzyme findings and our mRNA findings may be explained by the greater sensitivity and more quantitative nature of the TaqMan assay[39] compared to the protein assays or by the smaller number of samples studied for protein expression or activity compared to the 111 samples analyzed by RT-PCR.

Our results suggest that GSTPI mRNA expression levels offer more promise than GSTPI protein levels as biomarkers for following disease progression in individuals with Barrett's esophagus. It seems plausible that patients with Barrett's esophagus, who have a more abnormal GSTPI expression profile, are at greater risk of progression to higher disease stages because of the decreased capacity to detoxify carcinogens, but this needs to be demonstrated in studies of sequential biopsies in individual patients. It is likely that molecular diagnosis and staging of Barrett's esophagus will probably require the assessment of a panel of gene expressions. Studies from this institution and elsewhere suggest that many genes have significantly different expressions or mutation frequencies at different stages of Barrett's disease.[24,27,35–38]

GSTPI mRNA expression levels were significantly lower in normal squamous esophagus tissues from patients with cancer compared to patients with the maximum diagnosis of Barrett's esophagus, and the control group without evidence of Barrett's esophagus or chronic gastroesophageal reflux. We and others have found similar evidence of the presence of a widespread oncogenic "field effect" in the normal esophagus of cancer patients in studies of gene expression and DNA methylation analysis.[24,31,37,38–40] One explanation for this field change is that because of an injurious environmental agent, for example, the gastroesophageal refluxate, some of the early events of tumorigenesis have already occurred. These early events might predispose the apparently normal squamous esophagus tissue to undergo further genetic changes, leading ultimately to the development of Barrett's esophagus and adenocarcinoma. An alternative explanation is that clones of abnormal cells, in the presence of cancer, have expanded widely throughout the mucosa to replace previously normal cells. In either case it is apparent that genetic changes can precede the appearance of morphologic changes in this disease.

CONCLUSION

Using a fluorescent quantitative PCR method for GSTPI mRNA expression analysis, we detected significant alterations in gene expression for each stage of the Barrett's metaplsia-dysplasia-adenocarcinoma sequence. Loss of GSTPI mRNA and protein expression might be an important mechanism involved in the development and progression of this disease. Quantitation of GSTPI mRNA expression in patients with Barrett's esophagus might be a useful biomarker to identify patients at higher risk for progression to cancer.

REFERENCES

1. Devesa SS, Blot WJ, Fraumeni JF. Changing patterns in the incidence of esophageal carcinoma in the United States. Cancer 1998;83:2049–2053.
2. Lord RV, Law MG, Ward RL, Giles GG, Thomas RJ, Thursfield V. Rising incidence of oesophageal adenocarcinoma in men in Australia. J Gastroenterol Hepatol 1998; 83:2049–2053.
3. Blot WJ, McLaughlin JK. The changing epidemiology of esophageal cancer. Semin Oncol 1999;26:2–8.
4. Pera M, Cameron AJ, Trastek VF, Carpenter HA, Zinsmeister AR. Increasing incidence of adenocarcinoma of the esophagus and esophagogastric junction. Gastroenterology 1993;104:510–513.
5. Williamson WA, Ellis FH, Gibb SP, Shanian DN, Aretz HT, Heatly GJ, Watkins E. Barrett's esophagus: Prevalence and incidence of adenocarcinoma. Arch Intern Med 1991; 151:2212–2216.
6. Cameron AJ, Lomboy CT. Barrett's esophagus: Age, preva-

lence, and extent of the columnar epithelium. Gastroenterology 1992;103:1241–1245.
7. Barrett MT, Sanchez CA, Prevo LJ, Wong DJ, Galipeau PC, Paulson TG, Rabinovitch PS, Reid BJ. Evolution of neoplastic cell lineages in Barrett's esophagus. Nat Genet 1999;22:106–109.
8. Drewitz DJ, Sampliner RE, Garewal HS. The incidence of adenocarcinoma in Barrett's esophagus: A prospective study of 170 patients followed 4.8 years. Am J Gastroenterol 1997;92:212–215.
9. Hayes JD, Pulford DJ. The glutathione S-transferase supergene family: Regulation of GST and contribution of the isoenzymes to cancer chemoprevention and drug resistance. Crit Rev Biochem Mol Biol 1995;30:445–600.
10. Morrow CS, Cowan CH, Goldsmith ME. Structure of the human genomic glutathione S-transferase-π gene. Gene 1989;75:3–11.
11. Beckett GJ, Hayes JD. Glutathione S-transferases: Biomedical applications. Adv Clin Chem 1993;30:281–380.
12. Tsuchida S, Sato K. Glutathione transferases and cancer. Crit Rev Biochem Mol Biol 1992;27:337–384.
13. Peters WHM, Kock L, Nagengast FM, Kremers PG. Biotransformation enzymes in human intestine: Critical levels in the colon? Gut 1991;32:408–412.
14. Peters WHM, Nagengast FM, Van Tongeren JHM. Glutathione S-transferase, cytochrome P450, and uridine 5′diphosphate-glucuronosyltrasnferase in human small intestine and liver. Gastroenterology 1989;96:783–789.
15. Peters WHM, Roelofs HMJ, Hectors MPC, Nagengast FM, Jansen JBMJ. Glutathione and glutathione S-transferases in Barrett's epithelium. Br J Cancer 1993;67:1413–1417.
16. De Bruin WCC, Wagenmans MJM, Peters WHM. Expression of glutathione S-transferase α, P1-1 and T1-1 in the human gastrointestinal tract. Jpn J Cancer Res 2000;91: 310–316.
17. Seidegrad J, Pero RW, Markowitz MM, Roush G, Miller DG, Beattie EJ. Isoenzymes of glutathione S-transferases (class μ) as a marker for susceptibility for lung cancer: A follow-up study. Carcinogenesis 1990;11:33–36.
18. Chevenix-Trench G, Young J, Coggan M, Board P. Glutathione S-transferase M1, and T1 polymorphism: Susceptibility to colon cancer and the age of onset. Carcinogenesis 1995;16:1655–1657.
19. Deakin M, Elder J, Hendrickse C, Peckham D, Baldwin D, Pantin C, Wild N, Leopard P, Bell DA, Jones P, Duncan H, Brannigan K, Alldersea J, Fryer AA, Strange RC. Glutathione S-transferase GSTT1 genotype and susceptibility to cancer: Studies of interactions with GSTM1 in lung, oral, gastric and colorectal cancers. Carcinogenesis 1996; 17:881–884.
20. Van Lieshout EM, Tiemessen DM, Witteman BJM, Peters WHM. Low glutathione and glutathione S-tranferase levels in Barrett's esophagus as compared to normal esophagus epithelium. Jpn J Cancer Res 1999;90:81–85.
21. Compton KR, Orringer MB, Beer DG. Induction of glutathione S-transferase-π in Barrett's metaplasia and Barrett's adenocarcinoma cell lines. Mol Carcinog 1999;24: 128–136.
22. Ishioka C, Kanamaru R, Shibata H, Konishi Y, Ishikawa A, Wakui A, Sato T, Nishihira T. Expression of glutathione S-transferase-pi messenger RNA in human esophageal cancers. Cancer 1991;67:2560–2564.
23. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–159.
24. Lord RV, Salonga D, Danenberg KD, Peters JH, DeMeester TR, Park JM, Johannson J, Skinner KA, Chandrasoma P, DeMeester SR, Bremner CG, Tsai PI, Danenberg PV. Telomerase reverse transcriptase expression is increased early in the Barrett's metaplasia, dysplasia, carcinoma sequence. J GASTROINTEST SURG 2000;4:135–142.
25. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res 1996;6:986–994.
26. Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. Genome Res 1996; 6:995–1001.
27. Brabender J, Lord RV, Danenberg KD, Metzger R, Schneider PM, Uetake H, Kawakami K, Park JM, Salonga D, Peters JH, DeMeester TR, Hölscher AH, Danenberg PV. Upregulation of ornithine decarboxylase mRNA expression in Barrett's esophagus and Barrett's associated adenocarcinoma. J GASTROINTEST SURG 2001;5:174–182.
28. Rabinovitch PS, Reid BJ, Haggitt RC, Norwood TH, Rubin CE. Progression to cancer in Barrett's esophagus is associated with genomic instability. Lab Invest 1989;60:65–71.
29. Reid BJ, Blount PL, Rubin CE, Levine DS, Haggitt RC, Rabinovitch PS. Flow-cytometric and histological progression to malignancy in Barrett's esophagus: Prospective endoscopic surveillance of a cohort. Gastroenterology 1992; 102:1212–1219.
30. Reid BJ, Barrett MT, Galipeau PC, Sanchez CA, Neshat K, Cowan DS, Levine DS. Barrett's esophagus: Ordering the events that lead to cancer. Eur J Cancer Prev 1996;2:57–65.
31. Eads CA, Lord RV, Wickramasinghe K, Long TL, Kurumboor SK, Bernstein L, Peters JH, DeMeester SR, DeMeester TR, Skinner KA, Laird PW. Epigenetic patterns in the progression of esophageal adenocarcinoma. Cancer Res 2001;61:3410–3418.
32. Van Lieshout EM, Roelofs HM, Dekker S, Mulder CJ, Wobbes T, Jansen JB, Peters WH. Polymorphic expression of the glutathione S-transferase PI gene and its susceptibility to Barrett's esophagus and esophageal carcinoma. Cancer Res 1999;59:586–589.
33. Laird PW, Jaenisch R. The role of DNA methylation in cancer genetics and epigenetics. Annu Rev Genet 1996;30: 441–446.
34. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: A fundamental aspect of neoplasia. Adv Cancer Res 1998;72:141–196.
35. Schneider PM, Casson AG, Levin B, Garewal HS, Hoelscher AH, Becker K, Dittler HJ, Cleary KR, Troster M, Siewert JR, Roth JA. Mutations of p53 in Barrett's esophagus and Barrett's cancer: A prospective study of ninety-eight cases. J Thorac Cardiovasc Surg 1996;111:323–333.
36. Hayashi K, Metzger R, Salonga D, Danenberg K, Leichman LP, Fink U, Sendler A, Kelsen D, Schwartz GK, Groshen S, Lenz HJ, Danenberg PV. High frequency of simultaneous loss of p16 and p16beta gene expression in squamous cell carcinoma of the esophagus but not adenocarcinoma of the esophagus or stomach. Oncogene 1997;15: 1481–1488.
37. Lord RV, Tsai PI, Danenberg KD, Peters JH, DeMeester TR, Tsao-Wei DD, Groshen S, Salonga D, Park JM, Crookes PF, Kiyabu M, Chandrasoma P, Danenberg PV. Retinoic acid receptor-α messenger RNA expression is increased and retinoic acid receptor-γ is decreased in Barrett's intestinal metaplasia, dysplasia, adenocarcinoma sequence. Surgery 2001;129:267–276.
38. Brabender J, Lord RV, Danenberg KD, Metzger R, Schneider PM, Park JM, Salonga D, Groshen S, Tsai-Wei DD, DeMeester TR, Hölscher AH, Danenberg PV. Increased c-myb mRNA expression in Barrett's esophagus and Barrett's associated adenocarcinoma. J Surg Res 2001;99:301–306.

39. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, Danenberg PV, Laird PW. Methylight: A high-throughput assay to measure DNA methylation. Nucleic Acids Res 2000;28:E32.
40. Eads CA, Lord RV, Kurumboor SK, Wickramasinghe K, Skinner ML, Long TI, Peters JH, DeMeester TR, Danenberg KD, Danenberg PV, Laird PV. Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinomas. Cancer Res 2000;60:5021–5026.

Development of Achalasia Secondary to Laparoscopic Nissen Fundoplication

Nicholas Stylopoulos, M.D., Cheryl J. Bunker, M.D., David W. Rattner, M.D.

Dysphagia after laparoscopic Nissen fundoplication (LNF) is commonly attributed to edema and/or improperly constructed wraps, and in some instances the cause can be difficult to identify. We report, for the first time, the development of secondary achalasia after LNF as a cause of late-onset postoperative dysphagia. A total of 250 consecutive patients undergoing LNF were analyzed for the development of postoperative dysphagia at a university hospital. Patients were considered to have secondary achalasia if they met the following four criteria: (1) preoperative manometry demonstrating normal peristalsis and normal lower esophageal sphincter (LES) relaxation; (2) lack of esophageal peristalsis on postoperative manometry or fluoroscopy with or without incomplete LES relaxation; (3) no mucosal lesions seen on endoscopy; and (4) dysphagia refractory to dilatation. The following three groups of patients were identified: patients who developed secondary achalasia (group A, n = 7); patients with persistent dysphagia requiring and responding to postoperative dilatation (group B, n = 12 patients); and patients whose postoperative recovery was not complicated by dysphagia (group C, n = 231). The groups were comparable in terms of all preoperative variables except for age. Patients in group A were older than those in group B (57 years [range 27 to 66 years] vs. 36.5 years [range 27 to 63 years], $P = 0.028$) but were not significantly older than patients in group C (45 years [range 20 to 84 years], $P = 0.42$). The onset of severe dysphagia was later in group A than in group B (135 days [range 15 to 300 days] vs. 20 days [range 9 to 70 days], $P = 0.002$). The median weight loss in group A was also significantly greater than in Group B (15 pounds [range 11 to 44 pounds] vs. 4 pounds [range 0 to 15 pounds], $P = 0.0007$). Two patients in group A who underwent reoperation failed to improve. Botulinum toxin injections were tried in two patients and Heller myotomy in one with good results. Nine patients in group B improved promptly after one dilatation, and three improved after two dilatations. Secondary achalasia should be considered as one of the causes of persistent dysphagia after an apparently successful antireflux operation. Secondary achalasia tends to occur in older patients and is characterized by a delayed onset of symptoms. Imaging studies are a reliable means of excluding mechanical obstruction as a cause of secondary achalasia, and a negative result should raise the suspicion of secondary achalasia. Esophageal motility studies are necessary to confirm the diagnosis. Failure to consider the diagnosis of secondary achalasia can lead to multiple fruitless attempts at dilatation or even inappropriate reoperations. (J GASTROINTEST SURG 2002;6:368–378.)

KEY WORDS: Achalasia, dysphagia, fundoplication, gastroesophageal reflux disease

Dysphagia is a well-known complication of both open and laparoscopic antireflux operations. Postoperative dysphagia is usually transient, subsiding within several weeks of the operation as edema at the surgical site resolves. Esophageal dilatation may be necessary in 5% to 10% of patients to facilitate this process. A small subset of patients develops permanent dysphagia after antireflux surgery. Causes of permanent dysphagia include mechanical obstruction at the crural level and problems with placement or construction of the fundic wrap. Several investigators have reported hiatal fibrosis due to use of electrocautery or overly tight closure of the hiatus. Similarly, wraps that are constructed improperly or have slipped may also lead to nondilatable dysphagia.[1–6] In some instances, however, the cause of dysphagia that develops after an apparently technically successful operation can be difficult to identify. In our recent experience,

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (oral presentation).
From the Divisions of Gastroenterology (C.J.B.) and General and Gastrointestinal Surgery (D.W.R.), Department of Surgery (N.S.), Massachusetts General Hospital; and the Departments of Medicine (C.J.B.) and Surgery (D.W.R.), Harvard Medical School, Boston, Massachusetts.
Reprint requests: David W. Rattner, M.D., Chief, Division of General and Gastrointestinal Surgery, Massachusetts General Hospital, Boston, MA 02114. e-mail: drattner@partners.org

Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(02)00019-7

we encountered seven patients who developed late-onset dysphagia after laparoscopic Nissen fundoplication (LNF) and ultimately were found to have radiographic and manometric changes consistent with achalasia. The aim of this study was to report on the development of secondary achalasia after LNF and to identify those characteristics that distinguish secondary achalasia from routine post-Nissen dysphagia.

PATIENTS AND METHODS

A prospective database was maintained for all antireflux operations performed by a single surgeon at a university hospital. A total of 250 consecutive patients undergoing LNF were retrospectively analyzed for the development of postoperative dysphagia. The 105 men and 145 women had a median age of 42 years (range 20 to 84 years). The indication for surgery was symptomatic gastroesophageal reflux disease (GERD) that was unresponsive to medical therapy. Patients operated on for treatment of paraesophageal hernias were excluded from this series. All patients underwent manometry and upper gastrointestinal endoscopy preoperatively. Esophageal motility studies were performed using a water-perfused system (Synectics Medical, Stockholm, Sweden). Lower esophageal sphincter resting pressure and relaxation were calculated relative to intragastric pressure. The diagnostic evaluation also included 24-hour pH probe monitoring for patients without documented esophagitis (163 patients) and barium esophagograms in 130 patients. LNF was performed in a standardized fashion that included loose crural closure, which left 1.5 cm of free space in the hiatus (measured with a pneumoperitoneum of 15 mm Hg), routine division of the short gastric vessels, and creation of a 2 cm wrap over a 56 F dilator. Patients were discharged the morning after surgery and instructed to remain on a full liquid diet for 1 week. Diets were then advanced to solid food as tolerated. Patients with dysphagia (defined as the inability to tolerate soft solids) persisting for more than 4 weeks underwent barium esophagograms. Patients who had ongoing weight loss or anxiety were considered for esophageal dilatation. The following three groups of patients were retrospectively identified: group A = patients who developed secondary achalasia after LNF; group B = patients with persistent dysphagia requiring and responding to postoperative dilatation; and group C = patients whose postoperative recovery was not complicated by dysphagia. The preoperative characteristics of the three groups of patients are shown in Table 1.

Patients were considered to have secondary achalasia if they met the following criteria: (1) Preoperative manometry demonstrating normal peristalsis with normal mean amplitudes of contraction (>35 mm) both proximally and distally, and normal lower esophageal sphincter (LES) relaxation; (2) complete disappearance of esophageal peristalsis on postoperative manometry or fluoroscopy with or without incomplete LES relaxation; (3) no mucosal lesions seen on endoscopic examination; and (4) dysphagia refractory to dilatation with 18 mm esophageal balloon dilators.

Statistical Analysis

All statistical analysis was carried out with the use of the SPSS statistical computer program (SPSS for Windows version 10.0, SPSS, Inc., Chicago, Illinois). Data are expressed as medians with ranges when appropriate. Groups were compared by means of the Mann-Whitney U test and Fisher's exact test. A P value of less than 0.05 was considered statistically significant.

RESULTS

Statistical analysis showed that patient groups were comparable in terms of all preoperative variables (symptoms and test results) except for age (see Table 1). Patients who developed secondary achalasia (group A) were older than those who required dilatation for relief of dysphagia (group B) (57 years [range 27 to 66 years] vs. 36.5 years [range 27 to 63years], $P = 0.028$) but were not significantly older than patients with routine recovery (group C) (45 years [range 20 to 84 years], $P = 0.42$).

Group A

Seven patients had secondary achalasia. A brief case history of each patient is presented in Table 2. The median age was 57 years (range 27 to 66 years). All patients described a period with minimal or no dysphagia after the operation. All were able to ingest soft solids during the early postoperative period. However, all reported progressive difficulty swallowing such that dysphagia became severe enough to cause them to seek medical advice. Four patients had regurgitation of undigested food occurring most commonly at night. Five patients had respiratory symptoms, which included aspiration, hoarseness, persistent cough, and exacerbation of asthma. Three patients complained of pain that was described as heartburn (n = 2) or chest pain (n = 1). The median postoperative weight loss was 15 pounds (range 11 to 44 pounds). Two patients met the standard mano-

Table 1. Preoperative characteristics of patients in Groups A, B, and C

	Group A	Group B	Group C
Patients (n)	7	12	231
Male/female ratio (n)	2/5	6/6	97/134
Median age (yr)	57 (range 27–66)	36.5 (range 27–63)	45 (range 20–84)
Median BMI	27 (range 23–29)	26.2 (range 19–32)	28.4 (range 18.5–45)
Typical GERD symptoms (n)*	7	11	191
Atypical GERD symptoms (n)*	3	3	67
Preoperative dysphagia (n)	0	3	20
Hiatal hernia (n)	7	5	156
Barrett's esophagus (n)	2	2	35
Mean amplitude of distal esophageal peristaltic contractions preoperatively (mm Hg)	57	55	56
Mean LES resting pressure preoperatively (mm Hg)	16.8	13.3	14.0

BMI = body mass index; n = number of patients.
*Some patients had both typical and atypical GERD symptoms.

metric criteria for a diagnosis of achalasia (i.e., incomplete LES relaxation associated with esophageal body aperistalsis) (Fig. 1). In five patients, esophageal manometry demonstrated aperistalsis but apparent complete LES relaxation. Results of barium swallow tests were abnormal in all patients (Fig. 2).

The median duration from the date of surgery to reevaluation was 135 days (range 15 to 300 days). The onset of severe dysphagia in patients with secondary achalasia occurred later than in patients who required dilatation (135 days [range 15 to 300days] vs. 20 days [range 9 to 70 days), $P = 0.002$) (Fig. 3). The median weight loss in patients with secondary achalasia was significantly greater than in those requiring dilatation (15 pounds [range 11 to 44 pounds] vs. 4 pounds [range 0 to 15 pounds], $P = 0.0007$). Esophageal balloon dilatation to 18 mm in three cases and to 20 mm in four cases was unsuccessful in relieving dysphagia. One patient who underwent conversion from a Nissen fundoplication to a Toupet fundoplication failed to improve. However, botulinum toxin injection was tried in three patients and Heller myotomy in one with good results. The history of two patients in particular is noteworthy and merits separate mention.

Case 1. A 48-year-old man (patient 1 in Table 2) was referred for management of medically intractable gastroesophageal reflux disease. An upper gastrointestinal series demonstrated good peristaltic waves, free gastroesophageal reflux, and a small hiatal hernia. Preoperative manometry showed normal LES pressure and relaxation, and normal esophageal peristalsis. The patient underwent a successful LNF. Postoperatively he remained free of symptoms of gastroesophageal reflux disease for a period of 150 days. By the end of this period, however, the patient reported the new onset of dysphagia for solids, nocturnal regurgitation, aspiration, coughing, and choking after most meals. An upper gastrointestinal series showed a functional obstruction at the gastroesophageal junction with delayed emptying of barium and no peristaltic activity. Esophageal manometry confirmed incomplete relaxation of the LES and demonstrated only nonpropulsive contractions. Esophageal dilatation with an 18 mm balloon was subsequently performed, but no improvement was seen. A decision to take down the fundoplication and reconstruct a partial wrap was made, and 180 days after the original operation, a redo laparoscopic Toupet fundoplication was performed. At reoperation, the Nissen fundoplication was intact. There did not appear to be excessive fibrosis of the hiatus and, in fact, the hiatal diameter appeared to be ample with some mobility of the esophagus within it. At a follow-up 3 weeks after the reoperation, the patient reported that his swallowing was better than it had been preoperatively, but it was far from optimal. However, the dysphagia worsened again and it became apparent that the revision of the initial procedure had not relieved his symptoms. A repeat esophageal manometric study demonstrated findings consistent with achalasia (Fig. 4). No peristalsis was noted either on endoscopic or fluoroscopic examination, and a barium swallow showed a bird beak–like appearance of the distal esophagus. Botulinum toxin was then injected locally at the LES, and the dysphagia was transiently improved. The patient continued to complain of chest pain and respiratory symptoms, and therefore underwent a Heller myotomy with excellent relief of dysphagia.

Case 2. A 57-year-old woman (patient 2 in Table 2) was referred for evaluation and management of complications subsequent to a laparoscopic Nissen fundoplication performed at another hospital. She had

Table 2. Characteristics of patients with secondary achalasia

Patient	Age (yr)/ Sex	Preoperative symptoms	UGI and endoscopy	Postoperative symptoms	Postoperative UGI/Manometry	Intervention/Outcome
1	48/M	Heartburn, regurgitation, chest pain	HH/antritis	Dysphagia, chest pain, regurgitation, respiratory symptoms, 12-pound weight loss	Aperistalsis, dilation, delay in emptying of barium/ Aperistalsis, incomplete LES relaxation	Dilatation/NI Redo laparoscopic Toupet/NI Botulinum toxin/I Heller myotomy/I
2	57/F	Heartburn, regurgitation	HH Barrett's esophagus Antritis	Dysphagia, heartburn, nausea, diarrhea, 44-pound weight loss	Aperistalsis, dilatation, delay in emptying of barium/ Aperistalsis, incomplete LES relaxation	Redo open Nissen, Heinecke-Miculicz/NI Dilatation/NI Botulinum toxin/I
3	65/F	Heartburn, regurgitation	HH Barrett's esophagus	Dysphagia, heartburn, regurgitation, 15-pound weight loss	Aperistalsis, Dilatation/ Aperistalsis	Dilatation/NI
4	53/F	Heartburn, regurgitation, respiratory symptoms	HH	Dysphagia, respiratory symptoms, 11-pound weight loss	Aperistalsis, delay in emptying of barium/Aperistalsis	Dilatation/NI
5	66/F	Heartburn, regurgitation, respiratory symptoms	HH/antritis	Dysphagia, respiratory symptoms, 21-pound weight loss	Aperistalsis/ Aperistalsis	Dilatation/NI
6	27/F	Heartburn, regurgitation, respiratory symptoms	HH	Dysphagia, regurgitation, respiratory symptoms, 19-pound weight loss	Aperistalsis, dilatation, delay in emptying of barium/ Aperistalsis	Dilatation/NI Botulinum toxin/I
7	57/M	Heartburn, regurgitation	HH	Dysphagia, gas bloat syndrome, 15-pound weight loss	Aperistalsis, dilatation/ Aperistalsis	Dilatation/NI

HH = hiatal hernia; I = improvement; NI = no improvement; UGI = upper gastrointestinal series.

a long history of gastroesophageal reflux, and the diagnostic evaluation before her initial operation revealed Barrett's esophagus and a small hiatal hernia. Manometry demonstrated a low resting tone of the LES with normal relaxation and normal esophageal peristalsis. After LNF, the patient had mild dysphagia but was primarily troubled by nausea after eating. She also reported a 30-pound weight loss, anorexia, and frequent diarrhea. She underwent an upper gastrointestinal series, which demonstrated a small residual axial hiatal hernia, with an ulcer in the herniated cardia, as well as retained food in the gastric fundus and antrum. Results of esophagogastroduodenoscopy were consistent with gastroparesis; esophagogastroduodenoscopy confirmed the presence of retained food in the stomach after an overnight fast and showed gastritis with multiple superficial erosions at the diaphragmatic level of the hiatal hernia. Given these results, a decision was made to revise the fundoplication and perform a pyloroplasty. Thus 4 months after the original operation, the patient underwent an open redo Nissen fundoplication and a Heinecke-Mikulicz pyloroplasty. At reoperation the fundoplication was found to be completely herniated into the mediastinum, and neither the anterior nor the posterior vagus nerve could be identified. The hernia was reduced and the wrap was taken apart completely. No significant esophageal shortening or scarring was noted, and the crural defect was rather large. A 2 cm standard 360-degree Nissen fundoplication was constructed over a 56 F Maloney dilator, and a pyloroplasty was performed. Following discharge from the hospital, the patient had progressively worsening dysphagia and was only able to tolerate clear liquids. One month later, the patient continued to have dysphagia for solids and was reluctant to ingest significant amounts by mouth. This resulted in an additional 14-pound weight loss. A jejunostomy tube was placed and a new round of diag-

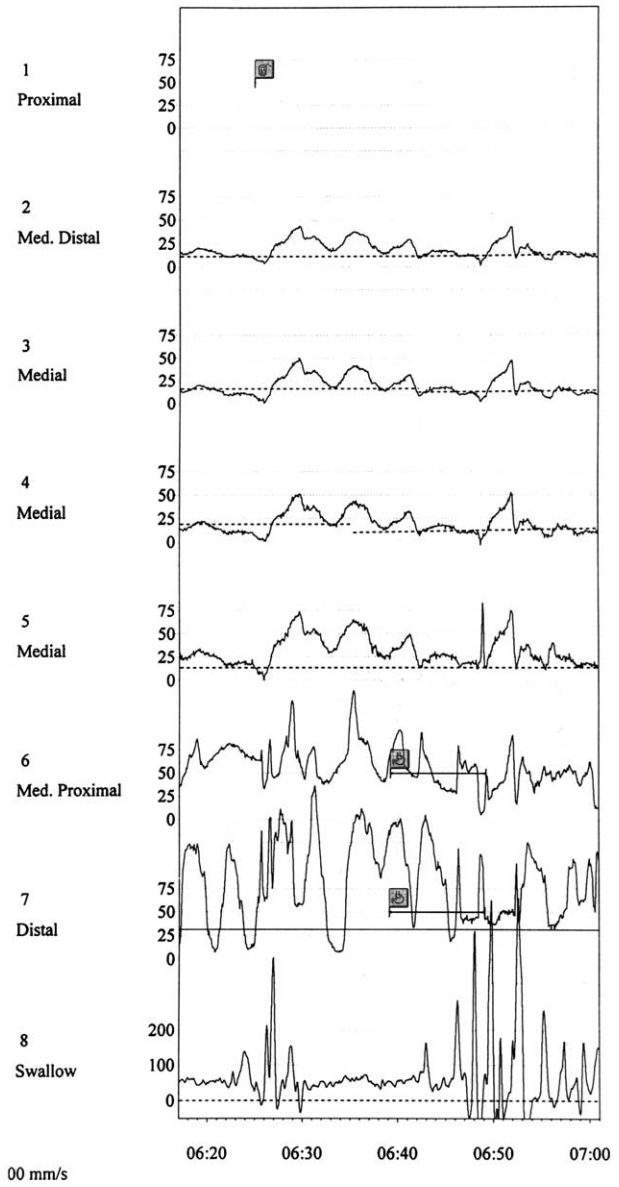


Fig. 1. Manometric tracing from a typical achalasia patient (not a postoperative patient from the current series). Ports 2 to 5 demonstrate simultaneous low-amplitude mirror-image contractions in response to the swallow. Port 6 shows a high resting pressure of the LES with no significant relaxation in response to a swallow.

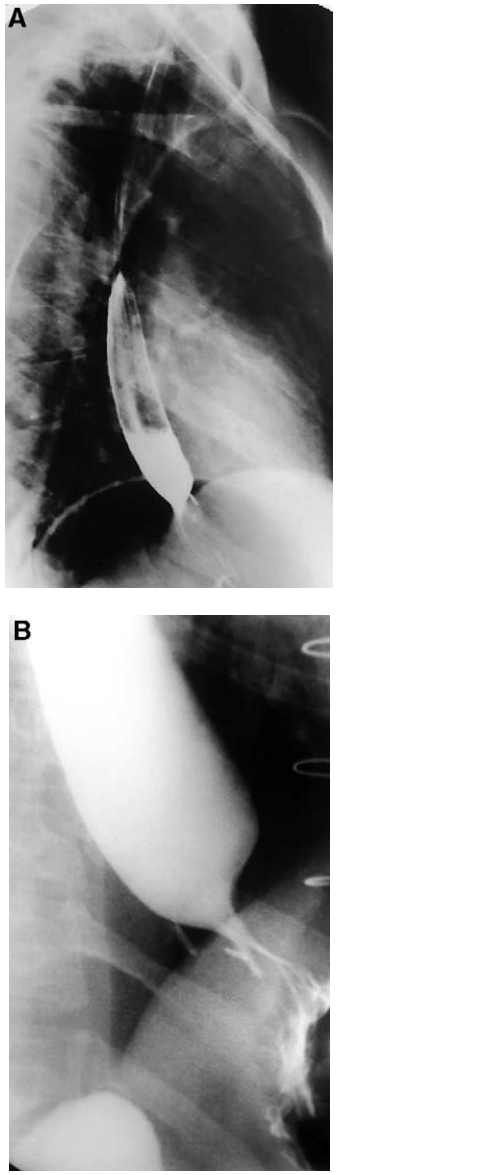


Fig. 2. A, Barium swallow of a patient with secondary achalasia (patient 6 in Table 2). Note the retained food and minimal dilatation of the esophagus. No peristalsis was observed during fluoroscopy. **B**, Barium swallow of a patient with early postoperative dysphagia that responded to dilatation. Note the narrowing at the site of the fundoplication and dilatation of the proximal esophagus.

nostic tests was initiated. An upper gastrointestinal series demonstrated dilatation of the esophageal body and complete disappearance of esophageal peristalsis. Manometry confirmed the absence of peristalsis and showed incomplete LES relaxation. The patient failed to respond to esophageal dilatation with the use of a 20 mm balloon dilator. Subsequently an intrasphincteric injection of botulinum toxin was administered, and the patient has had a good response to date.

Group B

The 12 patients in this group had either persistent dysphagia or severe early dysphagia, which required dilatation. The median age was 36.5 years (range 27 to 63 years). These patients uniformly had difficulty progressing from clear liquids to a full liquid and/or soft solid diet. Despite dietary modifications and other conservative measures, the dysphagia failed to

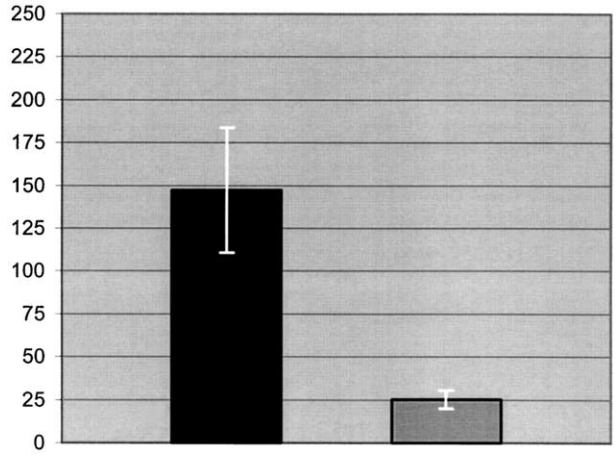


Fig. 3. Graphic representation of the time from surgery to the onset of dysphagia in patients with secondary achalasia (Group A, *left*) and patients with edema of the fundoplication that responded to dilatation (Group B, *right*). Patients with secondary achalasia had a delayed onset of dysphagia relative to patients whose dysphagia was presumably secondary to edema and responded to dilatation.

resolve. Esophageal peristalsis was evaluated by an upper gastrointestinal series and was found to be normal in all patients. Barium radiographs consistently demonstrated narrowing at the fundoplication site. These patients tended to be highly symptomatic and underwent endoscopy and balloon dilatation at a median of 20 days postoperatively (range 9 to 70 days). Nine of the 12 patients showed prompt improvement after one dilatation, whereas three improved after a second dilatation. No patient has subsequently complained of persistent dysphagia.

Group C

A total of 231 patients had postoperative recovery that was not complicated by clinically significant dysphagia.

DISCUSSION

Achalasia is a rare disorder characterized by almost complete disruption of normal esophageal motility. An epidemiologic study from Rochester, Minnesota has shown an incidence of 0.6 new cases per 100,000 population per year.[7] According to the Agency for Healthcare Research and Quality, achalasia was the chief reason for 2612 hospital admissions in 1997.[8] Secondary achalasia refers to achalasia that develops as a result of an underlying disorder. It has been associated with a variety of conditions, which are summarized in Table 3. The development of achalasia after surgery is a rare occurrence. Only 60 cases have been reported in the English literature since 1947,[9–28] and of these only 10 occurred after antireflux surgery[25–28] (Table 4). The current series is the first to report the development of secondary achalasia after LNF. Because antireflux operations are being performed more frequently in the laparoscopic era, it is likely that more cases of secondary achalasia will occur.

The pathophysiology of primary and secondary achalasia remains unknown, and a variety of neuropathic changes and physiologic abnormalities have been described.[29] Experimental models of achalasia have been produced by placing a constrictive band around the distal esophagus in cats.[30] This may be analogous to what has been described as iatrogenic achalasia.[25] In patients with iatrogenic achalasia, the loss of peristalsis and LES dysfunction is precipitated by mechanical narrowing of the region of the gastroesophageal junction, which is related to inflammation, edema, hematoma, fibrosis, or a tight fundoplication. The onset of dysphagia usually occurs in the early postoperative period, but it can also occur many years after the operation. Some patients respond to dilatation, but in the most patients takedown of the stenotic repair is required. Another category of patients are those with unrecognized preoperative primary achalasia who are mistakenly diagnosed and treated as having gastroesophageal reflux.[31,32] They should not be considered as having secondary achalasia, because the condition existed prior to antireflux surgery. These observations highlight the importance of routinely assessing esophageal motility preoperatively to rule out the presence of a primary esophageal motor disorder before performing antireflux surgery.

Poulin et al.[28] have proposed a classification of the achalasia syndromes that are seen after antireflux surgery based on the timing of the appearance of symptoms and the duration of the symptom-free interval. According to these investigators, secondary achalasia is the development of achalasia in the early postoperative period that is caused by fibrosis or stenosis at the operative site. Patients with late-onset achalasia after antireflux surgery, without evidence of mechanical obstruction, are classified as having metachronous primary achalasia. The term "primary" is used because the authors suggest that this syndrome cannot be demonstrated to be related to hiatal surgery. We believe that the interval between hiatal surgery and the development of achalasia is not a useful predictor of the pathogenic cause of achalasia and cannot preclude a possible causative relationship between antireflux surgery and achalasia. Because the incidence of achalasia in the general

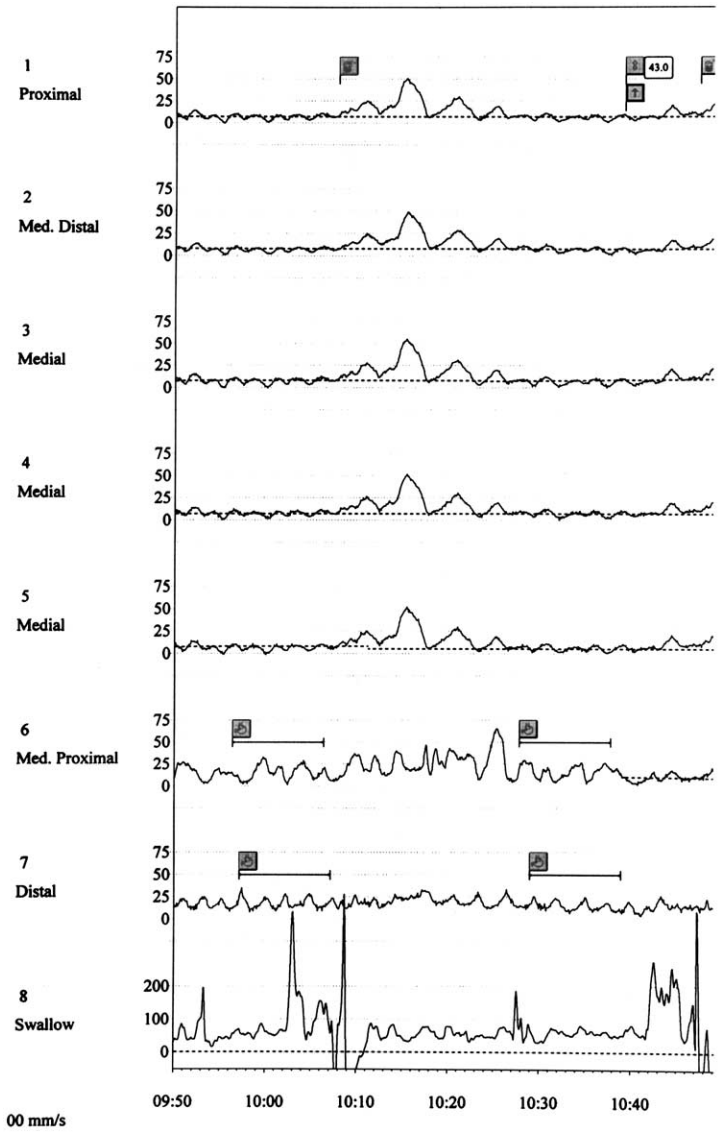


Fig. 4. Manometric tracing from patient 1 with findings of secondary achalasia. This tracing is remarkably similar to that shown in Fig. 1. Just as in classic achalasia, ports 2 to 5 demonstrate simultaneous mirror-image contractions in response to the swallow. Port 6 shows normal resting pressure of the LES, but with incomplete relaxation in response to a swallow.

population is low and the frequency of antireflux surgery in the population is also low, the association of LNF and secondary achalasia suggests that achalasia in these patients is related to surgery.

The precise mechanism for the development of secondary achalasia in our patients is unclear. LNF was performed in the same standardized fashion, and in six of seven patients the operation appeared to be a technical success. Postoperative barium radiographic and endoscopic findings ruled out mechanical reasons and technical errors (with the exception of patient 2). Two patients have undergone reoperation. In one of them, a vagal nerve injury was identified. At reoperation in both patients, the fundoplication did not appear to be tight and there was no evidence of excessive scar tissue formation either at the hiatus or the wrap. Because vagal injury was evident in only one patient, it is doubtful that vagal denervation of the esophagus was the only cause of secondary achalasia in this series. Postvagotomy achalasia is believed to be related to interruption of the vagal preganglionic fibers that supply the LES rather than the distal nerve segments. Furthermore, although vagotomy may occasionally be associated with transient postoperative dysphagia, it does not usually lead to the manometric features of classic achalasia.

Two of our patients met the standard manometric criteria for diagnosis of achalasia (i.e., incomplete relaxation of the LES associated with esophageal body

Table 3. Causes of secondary achalasia

Chagas' disease
Neoplasms of the distal esophagus and gastric cardia
Nongastrointestinal neoplasms
Hematologic malignancies
Amyloidosis
Connective tissue–autoimmune diseases
Rare familial and congenital disorders
Pancreatic pseudocyst
Surgery

aperistalsis), whereas five patients demonstrated aperistalsis but appeared to have nearly complete relaxation of the LES.[33–37] It has been postulated that these patients suffer from a somewhat milder impairment of LES function, and this manometric finding does not preclude the diagnosis of achalasia, provided that the typical symptoms and radiographic findings are seen. LES relaxation in patients with aperistalsis documented by manometry has been described as a marker for a subgroup of patients with an early stage of achalasia. In our study, patients with incomplete LES relaxation were diagnosed earlier, had a shorter symptom-free period, and showed a greater maximal weight loss compared to patients with complete LES relaxation.

The patients in this series had neither misdiagnosed primary achalasia nor iatrogenic achalasia. All patients had normal esophageal peristalsis preoperatively and normal relaxation of the LES. Cautery was not used in the hiatal dissection, and the loose crural closure always left adequate space around the esophagus. Multiple dilations with 18 mm and 20 mm dilators failed to improve dysphagia in the patients with secondary achalasia, whereas patients in group B with persisting postoperative edema uniformly responded to this treatment modality. Furthermore, dysphagia rarely began immediately postoperatively, and all patients treated with botulinum toxin thus far have responded. If the etiology of their dysphagia was due to a mechanical narrowing or fibrosis, the dysphagia would not be expected to respond to botulinum toxin.

The addition of our seven patients yields a total of 17 reported cases of secondary achalasia after antireflux surgery. In these patients, there is no apparent explanation for postsurgical achalasia. There is no evidence of fibrosis, scarring, or stenosis either on diagnostic studies or at reoperation. Patients usually have a long symptom-free period that may even last for years. Poulin et al.[28] described a patient who had undergone antireflux surgery at the age of 38 years. After a symptom-free interval, she gradually developed symptoms of dysphagia and underwent multiple dilatations. She was finally diagnosed and treated appropriately 31 years after the initial operation. These patients are unresponsive to dilatation, and revision of the initial procedure may fail to relieve dysphagia if the diagnosis of secondary achalasia has not been made. The administration of botulinum toxin has the same therapeutic effects and disadvantages in patients with secondary achalasia as it does in patients with primary achalasia. Esophageal myotomy has provided excellent results and is probably the optimal therapy once the diagnosis of secondary achalasia is made.

This study should alert surgeons to consider secondary achalasia as one of the causes of persistent dysphagia after an apparently successful antireflux operation. Failure to consider the diagnosis of secondary achalasia can lead to multiple fruitless attempts at dilatation or even inappropriate surgical conversion of LNF to a partial fundoplication. The clinical characteristics that distinguish secondary achalasia from routine post-Nissen dysphagia include occurrence in older patients and delayed onset of symptoms. All patients describe a period

Table 4. Reported cases of secondary achalasia after surgery

References	Operation	No. of patients	Proposed pathogenesis
9–24	Vagotomy	21	Inflammation, edema, hematoma, fibrosis
9–17	Truncal	15	
18–23	Selective	5	
24	Highly selective	1	
25	Hill procedure	6	Tight wrap/diaphragmatic closure
26	Pexy of the round ligament	2	Fibrosis/stricture
25	Aorta–superior mesenteric artery bypass graft (diaphragmatic closure)	1	Tight diaphragmatic closure
27	Radical gastrectomy	1	Tumor invasion of the myenteric plexus/vagus
28	Open Collis-Nissen gastroplasty	1	Not apparent
28	Open posterior fundoplication	1	Not apparent
Current series	Laparoscopic Nissen fundoplication	7	Not apparent

of relatively normal swallowing before the progressive and relatively surprising onset of severe dysphagia. Barium swallow and endoscopy are reliable means for excluding mechanical obstruction, and normal results of these tests should raise the suspicion of secondary achalasia. Esophageal motility studies are necessary to confirm the diagnosis when it is suspected. It has been stated that the surgical literature underreports the numerous gastrointestinal side effects that are commonly seen after fundoplications.[38] On the basis of our experience, we believe that secondary achalasia after surgery for GERD may be an underrecognized complication. With the increasing volume of fundoplications now being performed, this entity needs to be considered in the evaluation of postoperative dysphagia.

REFERENCES

1. Watson DI, Jamieson GG, Mitchell PC, Devitt PG, Britten-Jones R. Stenosis of the esophageal hiatus following laparoscopic fundoplication. Arch Surg 1995;130:1014–1016.
2. Devitt PG, Watson DI, Kennedy A, Game PA, Jamieson GG. Posterior versus anterior hiatal repair during laparoscopic Nissen fundoplication: A randomised controlled trial. Aust N Z J Surg 1999;69:57–58.
3. Hunter JG, Smith CD, Branum GD, Waring JP, Trus TL, Cornwell M, Galloway K. Laparoscopic fundoplication failures: Patterns of failure and response to fundoplication revision. Ann Surg 1999;230:595–606.
4. Soper N, Dunnegan D. Anatomic fundoplication failure after laparoscopic antireflux surgery. Ann Surg 1999;229:669–677.
5. Horgan S, Pohl D, Bogetti D, Eubanks T, Pelligrini C. Failed antireflux surgery: What have we learned from reoperations? Arch Surg 1999;134:809–817.
6. Low DE, Mercer CD, James EC, Hill LD. Post Nissen syndrome. Surgery 1988;167:1–5.
7. Earlam RJ, Ellis FH Jr, Nobrega FT. Achalasia of the esophagus in a small urban community. Mayo Clin Proc 1969;44:478–483.
8. HCUPnet, Healthcare Cost and Utilization Project. Agency for Healthcare Research and Quality, Rockville, Maryland. http://www.ahrq.gov/data/hcup/hcupnet.htm
9. Grimson KS, Baylin CJ. Transthoracic vagotomy. JAMA 1947;134:925–932.
10. Carter SL. Resolution of postvagotomy dysphagia. JAMA 1978;240:2656–2657.
11. Sharp JR. Mechanical and neurogenic factors in postvagotomy dysphagia. J Clin Gastroenterol 1979;1:321–324.
12. Greatorex RA, Thorpe JA. Achalasia-like disturbance of oesophageal motility following truncal vagotomy and antrectomy. Postgrad Med J 1983;59:100–103.
13. Spencer JD. Postvagotomy dysphagia. Br J Surg 1975;62: 354–355.
14. Moses WR. Critique on vagotomy. N Engl J Med 1947;237:603–608.
15. Postlethwait RW, Kim SK, Dillon ML. Esophageal complications of vagotomy. Surg Gynecol Obstet 1969;128:481–488.
16. Harris J, Miller CM. Cardiospasm following vagotomy. Surgery 1960;47:568–570.
17. Wilcox RS. Cardiospasm following vagotomy. Am J Surg 1950;72:843.
18. Pierandozzi JS, Ritter JH. Transient achalasia. A complication of vagotomy. Am J Surg 1966;111:356–358.
19. Guelrud M, Zambrano-Rincones V, Simon C, Gomez G, Salinas A, Toledano A, Rudick J. Dysphagia and lower esophageal sphincter abnormalities after proximal gastric vagotomy. Am J Surg 1985;149:232–235.
20. Edwards DA. Post-vagotomy dysphagia. Lancet 1970;1:90–92.
21. Suleiman SI, Maglad SA, Hobsley M. Dysphagia following selective vagotomy. Br J Surg 1979;66:607–608.
22. Sapounov S, Sraieb R. Rare complications after selective vagotomy and pyloroplasty. Gastroplegia and achalasia of the cardia. J Radiol Electrol Med Nucl 1972;53:657–660.
23. Wirthlin LS, Malt RA. Accidents of vagotomy. Surg Gynecol Obstet 1972;135:913–916.
24. Duntemann TJ, Dresner DM. Achalasia-like syndrome presenting after highly selective vagotomy. Dig Dis Sci 1995;40:2081–2083.
25. Ellingson TL, Kozarek RA, Gelfand MD, Botoman AV, Patterson DJ. Iatrogenic achalasia. A case series. J Clin Gastroenterol 1995;20:96–99.
26. Parrilla P, Aguayo JL, Martinez de Haro L, Ortiz A, Martinez DA, Morales G. Reversible achalasia-like motor pattern of esophageal body secondary to postoperative stricture of gastroesophageal junction. Dig Dis Sci 1992;37:1781–1784.
27. Yeoman LJ, Grundy A, Parker MC, Fiennes AG. Pseudoachalasia after radical gastrectomy. Br J Surg 1989;76:97–98.
28. Poulin EC, Diamant NE, Kortan P, Seshadri PA, Schlachta CM, Mamazza J. Achalasia developing years after surgery for reflux disease: Case reports, laparoscopic treatment, and review of achalasia syndromes following antireflux surgery. J GASTROINTEST SURG 2000;4:626–631.
29. Reynolds JC, Parkman HP. Achalasia. Gastroenterol Clin North Am 1989;18:223–255.
30. Little AG, Correnti FS, Calleja IJ, Montag AG, Chow YC, Ferguson MK, Skinner DB. Effect of incomplete obstruction on feline esophageal function with a clinical correlation. Surgery 1986;100:430–436.
31. Mattox HE III, Albertson DA, Castell DO, Richter JE. Dysphagia following fundoplication: "Slipped" fundoplication versus achalasia complicated by fundoplication. Am J Gastroenterol 1990;85:1468–1472.
32. O'Brien CJ, Collins JS, Collins BJ, McGuigan J. Aperistaltic oesophageal disorders unmasked by severe post-fundoplication dysphagia. Postgrad Med J 1990;66:1047–1049.
33. Katz PO, Richter JE, Cowan R, Castell DO. Apparent complete lower esophageal sphincter relaxation in achalasia. Gastroenterology 1986;90:978–983.
34. Vantrappen G, Janssens J, Hellemans J, Coremans G. Achalasia, diffuse esophageal spasm, and related motility disorders. Gastroenterology 1979;76:450–457.
35. Mearin F, Malagelada JR. Complete lower esophageal sphincter relaxation observed in some achalasia patients is functionally inadequate. Am J Physiol Gastrointest Liver Physiol 2000;278:376–383.
36. Aliperti G, Clouse RE. Incomplete lower esophageal sphincter relaxation in subjects with peristalsis: Prevalence and clinical outcome. Am J Gastroenterol 1991; 86:609–614.
37. Hirano I, Tatum RP, Shi G, Sang Q, Joehl RJ, Kahrilas PJ. Manometric heterogeneity in patients with idiopathic achalasia. Gastroenterology 2001;120:789–798.
38. Swanstrom L, Wayne R. Spectrum of gastrointestinal symptoms after laparoscopic fundoplication. Am J Surg 1994;167:538–541.

Discussion

Dr. P. Barham: (Bristol, U.K.): I am not sure if I got this right. Were you investigating these patients 4 weeks after surgery?

Dr. D.W. Rattner: Yes, in persons who were unable to ingest soft solids and who were losing weight, we obtained a barium swallow.

Dr. Barham: So, this treatment was all carried out well before a year after their operations?

Dr. Rattner: Yes.

Dr. Barham: Because dysphagia will improve up to 6 months after surgery, and it is a common finding in the presence of an obstructed esophagus, either because of a tumor or some other mechanism, you will notice a pattern of achalasia. So you may be treating the early postoperative results rather than the true development of achalasia. Obstructed bowel will show simultaneous contractions.

Dr. Rattner: That is correct. I think your concern is one that we share; however, endoscopically there is no evidence of obstruction. Furthermore, these patients have a delayed onset of dysphagia. These patients were dysphagia free until approximately 4 months after surgery, and some of them presented as late as 11 and 12 months. So I think primary obstruction is unlikely to be the cause.

Dr. J.H. Peters (Los Angeles, CA): I have been impressed by the number of patients who, if they are studied on postoperative day 2 or 3, have an aparistaltic dilated esophagus, but I would agree, this is different. My question is stimulated by a patient that I am currently treating who is struggling with this—a radiologist, actually. What is the natural history of this disease? Did you have any patients whom you did not treat, and can you give me some assurance that they are going to get better?

Dr. Rattner: We have four patients who have refused further intervention and who are struggling along. None of them were really able to resume eating solid food; none of them have gotten back to their preoperative weight. They are not particularly happy, and they have refused further intervention.

Dr. J.G. Hunter (Portland, OR): We have discussed several of these patients via e-mail. Our problem was that when we started performing Heller myotomies in patients with postfundoplication achalasia, they developed rather significant reflux postoperatively, so I discontinued that practice and started converting these patients to a Toupet fundoplication. We are really dealing with just a small body of experience, and I am not sure we have the answer. We should look at results of histologic examinations to see whether the pathologic findings of achalasia are present. In lieu of that, I think the other thing we could look at is postoperative motility to see if this meets the criteria for a variation of Koch's postulates. If this is true achalasia, then peristalsis should not return after a Heller myotomy; if this is outflow obstruction, there may be some return of peristalsis. Have you done any motility studies in these patients?

Dr. Rattner: I am sorry I did not make that clear. All seven have undergone postoperative motility testing, and the motility studies, in general, were done between 4 and 8 months after surgery. All have showed absence of peristalsis or mirror-image nonconducted contractions.

Dr. R. Onders (Cleveland, OH): I think Dr. Hunter was right on target with his point about the pathology of achalasia. I am able to routinely obtain a biopsy specimen of the esophageal muscle during a myotomy for pathologic diagnosis. Have you done that in these patients and will you perform a biopsy in the future? This will be the key in determining whether this is secondary or primary achalasia based on the inflammation of the ganglion cells in the biopsy specimen.

Dr. Rattner: We have not but I will. I cannot imagine that this is primary achalasia, because it is a rare disease, and so it is unlikely that we would have come into a cohort. There is a very good paper, if you were interested in reading more about this, that was published in the JOURNAL OF GASTROINTESTINAL SURGERY and presented as a poster at the 2000 SSAT meeting describing similar results. But none of the patients here had achalasia preoperatively. They all had normal preoperative manometric findings.

Dr. R. Bell (Englewood, CO): The question that you did not answer obviously is patients did not have manometric criteria for achalasia preoperatively, but didn't all of them have pH testing? Did you go back and look at those who had pH testing to make sure none of them had pseudoreflux? What percentage of these patients did not have a pH test? I think those are all basic questions that I am sure you have studied but need to be answered.

Dr. Rattner: I cannot tell you the answer off the top of my head as to what percentage of these patients did not have pH tests. They all either had documented esophagitis on biopsy or heartburn that responded to protein pump inhibitors.

Invited Discussion—Expert Commentator

Tom R. DeMeester, M.D. (Los Angeles, CA): This paper was excellent. The authors defined this new problem, secondary achalasia. These patients had preoperative normal motility; a symptom-free period, which I think is an extremely important observation; and then no mechanical cause for dysphagia. But that may be a little difficult to know. And in a laboratory, if you put a ribbon around the lower esophagus so that the length of the ribbon equals 110% of the circumference of the sphincter that you put it around, it will induce dysphagia and it will induce obstruction, because you have to allow for compliance as the bolus goes through, and if you interrupt compliance, you can cause dysphagia. So I am not sure we can be absolutely sure there is no outflow obstruction in patients who don't have any anatomical evidence for it.

What is really important to look at is whether there is a bolus pressure in the distal esophagus trying to show that there is greater pressure pushing the bolus through the sphincter area or not. That would be important to determine. I think it is important to realize that we can all miss achalasia. It often will present as heartburn early on, so you have to be careful.

I suspect that the cause for this problem is vagal injury. More and more we are realizing that vagal injury following this kind of operation can be a problem. You say vagal injury from what? Perhaps ischemia, operative trauma, or maybe stretch injury, just like you get with the brachial plexus. So we may be inducing it.

There was a period of time, at least in my residency, when we did a lot of ulcer surgery using vagotomy and pyloroplasty. We had what we called postvagotomy dysphagia—some of the old-timers will remember that. It occurred in patients, and it looked for all the world like achalasia. It didn't respond to dilatations, and it was a problem. I think secondary achalasia is probably the result of vagal injury.

Specific Reversible Stimulation of System y^+ L-Arginine Transport Activity in Human Intestinal Cells

Ming Pan, M.D., Ph.D., Wiley W. Souba, M.D., Sc.D., Anne M. Karinch, Ph.D., Cheng-Mao Lin, Ph.D., Bruce R. Stevens, Ph.D.

L-Arginine, which is intimately involved in cellular immune functions and nitric oxide biology, is transported by intestinal cells largely via transport System y^+. The gut epithelium is exposed to various luminal amino acids at any given time, and therefore the purpose of this study was to study the regulation of luminal arginine transport by other amino acids. System y^+ L-arginine transport activity was measured in Caco-2 monolayers exposed to various amino acids. L-arginine and/or other System y^+ substrates specifically upregulated System y^+ transport activity twofold after 1 hour, with a response noted as early as 5 minutes. Non–System y^+ substrates did not affect L-arginine absorption. Kinetic analysis indicated that L-arginine exposure increased both System y^+ K_m and V_{max}. Neither cycloheximide nor actinomycin affected this stimulation, indicating that the regulation did not involve transcription or translation. The System y^+ substrate activation effect was reversible. L-arginine transport activity returned to baseline within 3 hours when cells were reincubated in amino acid–free media. These data indicate that System y^+ arginine transport activity is rapidly and reversibly activated by System y^+ substrates via a mechanism consistent with transmembrane stimulation. These findings identify a mechanism by which luminal nutrients regulate arginine uptake by the gut. (J GASTROINTEST SURG 2002;6:379–386.)

KEY WORDS: Intestine, arginine, adaptive regulation

In humans, L-arginine is a conditionally essential amino acid during periods of rapid growth and development and after acute trauma.[1–3] Arginine absorbed from the intestinal lumen is the starting point for a variety of nitrogen intermediary metabolism reactions within enterocytes[4]; the mucosa supplies the portal vein with the appropriate metabolites or free arginine. Recent clinical trials have demonstrated that enteral arginine-supplemented nutrition improves immune functions and reduces septic complication and hospital costs.[5–15] Intestinal epithelia encounter varying luminal concentrations of various amino acids at any given time. In contrast to cells of internal organs that downregulate amino acid transport capability in response to specific substrates,[16,17] studies in the whole animal suggest that in response to augmented or reduced loads of specific amino acids, the small intestine selectively increases or decreases the specific substrate uptake ability.[18–24] However, studies are lacking regarding substrate-regulated intestinal amino acid transport activity at the cellular level in well-defined conditions of in vitro cell cultures.

The human intestinal epithelial cell line used in this study, Caco-2, is widely accepted as an in vitro model for transport studies of the small intestinal epithelium. The uptake of glucose, cationic and neutral amino acids, and other solutes in this cell line is comparable to uptake phenomena occurring in the intact small intestine.[25–28] We have previously characterized L-arginine transport systems in apical membranes during Caco-2 differentiation, and we have shown that arginine is transported predominantly by sodium-independent mechanisms.[29] The major pathways are denoted System y^+ (70%) and System y^+L or $b^{0,+}$ (30%). In this study we explored the effects of individual amino acids on the System y^+ activities in differentiated and undifferentiated Caco-2 cells. System y^+ is functionally defined as the sodium-independent, leucine-insensitive component of L-arginine uptake. Results of the present study enhance our understanding of intestinal arginine absorption regulations and provide the scientific foundation for managing enteral nutritional support of critical patients.

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia., May 20–23, 2001 (poster presentation).

From the Department of Surgery (M.P., W.W.S., A.M.K., C.-M.L.), Penn State College of Medicine, Milton S. Hershey Medical Center, Hershey, Pennsylvania; and the Department of Physiology (B.R.S.), College of Medicine, University of Florida, Gainesville, Florida.
Reprint requests: Ming Pan, M.D., Ph.D., Department of Surgery, H149, Penn State College of Medicine, MC 850, Hershey, PA 17033.

1091-255X/02/$—see front matter
PII: S1091-255X(01)00047-6

MATERIAL AND METHODS

Caco-2 Cell Cultures

The established human intestinal epithelial Caco-2 cell line was obtained from American Type Culture Collection (Rockville, Md.) at passage 16. Cells were grown in a humidified incubator at 37° C in 10% CO_2/90% O_2. Cells were routinely grown in Dulbecco's modified Eagle medium (DMEM) containing 25 mmol/L glucose, 4 mmol/L glutamine, 10% fetal bovine serum, 0.4 mol/L sodium bicarbonate, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1% nonessential amino acids. Caco-2 cells were passaged weekly after treatment with 0.05% trypsin and 0.02% EDTA. Cells were reseeded at a density of 4.5×10^6 cells per 100 mm dish for future subculturing, or seeded in the six-well cluster Falcon tissue culture dishes at a density of 4×10^5 cells per 35 mm well for transport experiments. Cells (passage 19 to 40) were used for experiments. The day of seeding was designated as day 0. The growth medium was changed daily, and cultures were inspected daily using a phase-contrast microscope.

Cell Treatments

All Caco-2 cells were incubated in arginine-free medium for 3 hours, and then incubated in the same medium containing various concentrations of alanine, arginine, or other amino acids ± cycloheximide (10 μmol/L) for various periods of time (30 seconds up to 48 hours) in a 37° C 10% CO/90% air-humidified incubator. The incubation medium was changed every 6 hours. The arginine-free medium contained 1.8 mmol/L $CaCl_2$, 0.2 μmol/L ferric nitrate, 0.4 mmol/L $MgSO_4$, 4.7 mmol/L $NaHCO_3$, 110 mmol/L NaCl, 1 mmol/L NaH_2PO_4, 25 mmol/L glucose, 0.04 mmol/L phenol Red·Na, 0.03 mmol/L choline chloride, 0.01 folic acid, 0.07 mmol/L myoinositol, 0.03 mmol/L niacinamide, 0.017 mmol/L D-pantothenic acid, 0.02 mmol/L pyridoxal·HCl, 1 μmol/L riboflavin, and 0.01 mmol/L thiamine·HCL. Caco-2 cells remained healthy (viability >99% by dye exclusion) during at least 48 hours of exposure to amino acid–free media.

Arginine Transport Measurements

Uptake of arginine was measured in cells 2 days after seeding (undifferentiated) and 9 days after seeding (differentiated) using cells started from the same seeding parent cells as previously described. System y^+ transporter activity was measured at room temperature (23° ± 1.0° C) for various periods of time (0 to 30 minutes). System y^+ is defined as sodium-independent, 10 mmol/L leucine-insensitive uptake of L-[^{3}H]arginine, as described in Caco-2 by us. After pretreatment of cells with individual amino acids, the medium was aspirated and cells were rinsed three times with uptake buffer (23° C) containing 137 mmol/L choline chloride, 10 mmol/L HEPES/Tris buffer (pH 7.4), 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, and 2.5 mmol/L $CaCl_2$. The uptake was initiated by adding 1 ml uptake buffer containing L-[^{3}H]arginine (2 μCi/ml, 0.5 μmol/L to 10 mmol/L unlabeled arginine), and 10 mmol/L L-leucine (10 mmol/L mannitol as control). Culture dishes were continuously shaken by an orbital shaker (1 Hz) during the uptake period (0 to 30 minutes). Uptake was arrested by aspirating the uptake buffer and immediately washing it three times with ice-cold buffer lacking substrate. At the end of each uptake period, the radioactivity of the isotope trapped in the cells was extracted by lysing cells with 1 ml of 1N NaOH that was neutralized with acetic acid. The radioactivity of the isotope was then assayed by liquid scintillation spectrometry. Protein in the NaOH extract was measured using the Bio-Rad protein assay (Hercules, California). Arginine uptake in both day 2 and day 9 cells was linear up to 10 minutes, and a 2-minute uptake period was selected for all the experiments in this study with zero time points serving as blanks. Uptake rates are expressed as moles of arginine per minute per milligram of cell protein. These uptake values represented steady-state isotope values. Sodium-dependent absorption of arginine and other amino acids (e.g., glutamine and alanine) was intact in both control and arginine-free medium groups. Uptake via the paracellular pathway in confluent cells (postseeding day 9) was minimal (<5%, n = 6), as confirmed by [^{3}C]-inulin extracellular studies (n = 6) and transepithelial arginine transport (n = 9; unpublished data).

Statistical Analysis

Each transport experiment was conducted in triplicate (including the zero-time blanks) in the same patch of cells, and all experiments were confirmed using at least two independent generations of cells (same postseeding days). Experimental results are reported as means ± SEM. Comparisons of means were made by analysis of variance with pairwise multiple comparisons by the Newman-Keuls method at $P < 0.05$. Transport kinetic parameters were obtained by fitting data to the Michaelis-Menten equation by nonlinear regression analysis using the Enzfitter computer program (Biosoft, Cambridge, U.K.).

RESULTS

System y^+ Activity Decreased in Amino Acid–Depleted Caco-2 Cells

The Caco-2 cells (day 2 and day 9) were incubated in the arginine-free medium with and without 1 mmol/L L-arginine or D-arginine for various length of time (30 seconds to 48 hours) in a 37° C/10% CO_2 incubator. Mannitol (1 mmol/L) was used as a control medium. The medium was changed every 6 hours to ensure that the amino acid concentration was constant and the possible autocrine accumulation was eliminated. The System y^+ transport activities were measured immediately after each incubation period. The System y^+ arginine (5 μmol/L) uptake activity decreased as the incubation time in arginine-free medium increased and reached the lowest level at approximately 3 hours' incubation where it remained for 48 hours (Fig. 1). Three hours' depletion incubation was chosen for the subsequent experiments. The day 2 and day 9 cells showed the same pattern of declining System y^+ activity in amino acid–free medium, with day 9 cells having lower baseline activity (see Fig. 1).

Decreased System y^+ Arginine Uptake Prevented by L- and D-Arginine Was Time and Dose Dependent

The declining System y^+ activity was prevented by adding L-arginine or D-arginine in the arginine-free medium. Adding L-arginine and D-arginine (at lesser degrees) into arginine-free medium in cells without previous amino aid depletion elevated the arginine transport activity staring at 30 minutes' incubation and continued to increase transport activity, which lasted for at least 48 hours (Fig. 2). To assess the how quickly System y^+ activity responds to substrate stimulation, Caco-2 cells were preincubated in arginine-free medium for 3 hours, and 1 mmol/L arginine was then added to the medium. The System y^+ arginine uptake increase was noticed as early as 5 minutes of arginine exposure.

To determine whether this arginine-induced System y^+ uptake was dependent on the amount of arginine presented, Caco-2 cells were incubated in arginine-free medium with L-arginine of various concentrations (0, 0.1 mmol/L, 1 mmol/L, and 10 mmol/L). The System y^+ activity induced by 1 mmol/L L-arginine was significantly higher than that induced by 0.1 mmol/L L-arginine, but was not significantly higher than that induced by 10 mmol/L L-arginine (Fig. 3). An L-arginine concentration of 1 mmol/L was chosen for subsequent experiments.

Pattern of System y^+ Activity Stimulated by Short-Term Exposure to Amino Acids

Initially we showed that arginine itself stimulated its own System y^+ activity. Next we examined the ef-

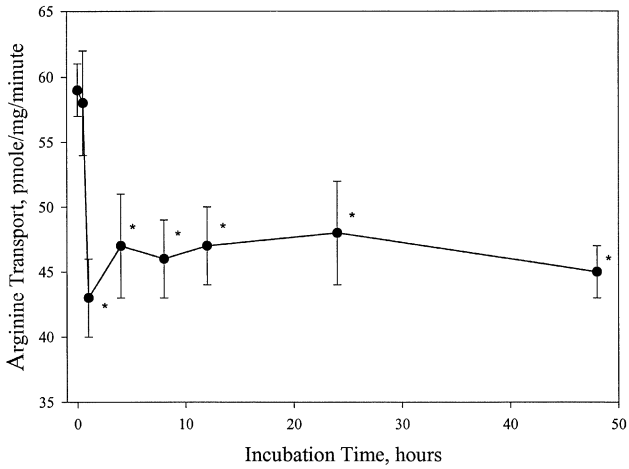


Fig. 1. System y^+ [^{3}H]-L-arginine transport activity in arginine-free medium. Uptake of leucine–insensitive [^{3}H]-L-arginine (5 μmol/L) was measured on day 2 in cells incubated in arginine-free medium for various periods of time (5 minutes to 48 hours). The arginine uptake was significantly lower starting at 1 hour of incubation, reached its lowest level at 3 hours of incubation, and lasted for at least 48 hours. A similar pattern was observed in day 9 cells; transport activity decreased to baseline 15 pmol/mg protein/minute from 25 pmol/mg protein/minute (data not shown). Transport values are means ± SEM (n = 9). *$P < 0.01$ vs. control.

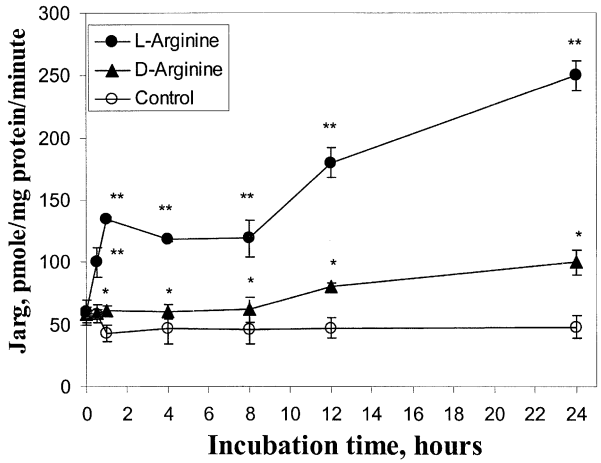


Fig. 2. Effect of L-arginine and D-arginine on System y^+ [^{3}H]-L-arginine transport activity in arginine-free medium. Uptake of [^{3}H]-L-arginine (5 μmol/L) was measured in day 2 cells incubated in arginine-free medium, arginine-free medium plus L-arginine (1 mmol/L), and arginine-free medium plus D-arginine (1 mmol/L) for up to 48 hours. Uptake in cells exposed to both L- and D-arginine was significantly greater than uptake in cells in arginine-free medium only. Transport values are means ± SEM (n = 9). *$P < 0.05$ vs. control; **$P < 0.01$ vs. control.

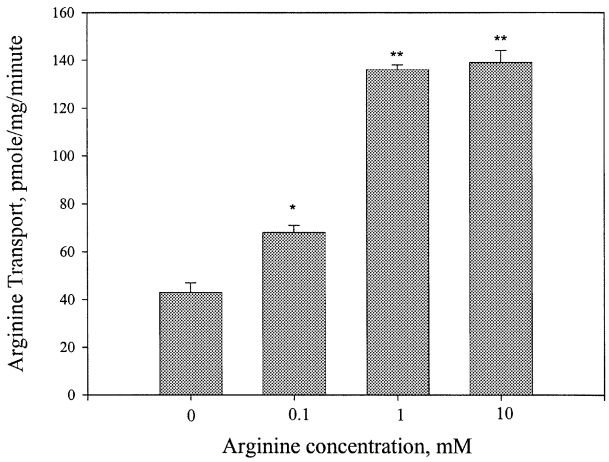


Fig. 3. Arginine stimulation of System y^+ activity was dose dependent. Uptake of [^{3}H]-L-arginine (5 μmol/L) was measured in cells incubated in arginine-free medium supplemented with various concentrations of L-arginine (0, 0.1, 1 or 10 mmol/L). Transport values are means ± SEM (n = 9). *$P <$ 0.05 vs. control; **$P < 0.001$ vs. control.

fect of individual amino acids on the System y^+ arginine transport activity. The Caco-2 cells were incubated in the arginine-free medium containing 1 mmol/L individual amino acids (1 mmol/L mannitol as control), with the exception of cysteine, which contained 1 mmol/L dithiothreitol as an antireducing agent (1 mmol/L dithiothreitol as control), for 3 hours. The System y^+ substrates lysine, arginine, and ornithine each exhibited robust stimulation of System y^+ activity (twofold increase). On the other hand, non– System y^+ substrates proline, 2-amino-2-norbornane-carboxylic acid, and alanine did not significantly affect the System y^+ activity. A pattern emerged such that the degree of arginine transport stimulation correlated with the inhibition pattern. Those amino acids that are weak inhibitors of System y^+ weakly stimulated System y^+ activity (Fig. 4). These data suggested that the System y^+ activity was specifically stimulated by its own substrates.

System y^+ Activity Increased by Acute Arginine Exposure Did Not Involve De Novo Protein Synthesis or Transcription

To determine whether protein synthesis or transcription was involved in System y^+ substrate stimulation, the Caco-2 cells (day 2 and day 9) were incubated in the arginine-free medium containing 1 mmol/L arginine (mannitol as control) ± 50 μmol/L cycloheximide or 0.5 μg/ml actinomycin D for 3 hours. Neither cycloheximide nor actinomycin D affected arginine-induced System y^+ activity (Fig. 5).

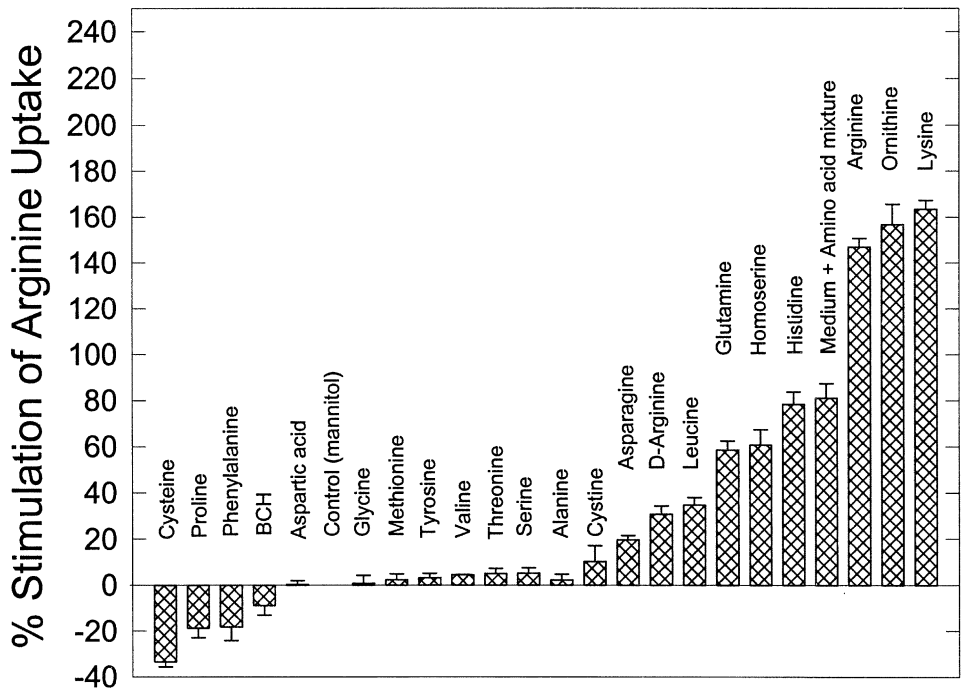


Fig. 4. Stimulation pattern of System y^+ L-arginine uptake by amino acids in arginine-free medium. Uptake of [^{3}H]-L-arginine (5 μmol/L) was measured in day 2 cells incubated in arginine-free medium supplemented with various amino acid analogues (1 mmol/L). Similar results were seen in day 9 cells (data not shown). Transport values are means ± SEM (n = 9).

Specific protein kinase C inhibitor chelerythrine chloride did not affect either the baseline or induced System y^+ arginine transport.

System y^+ Activity Increased by Acute Arginine Exposure Was Reversible

Caco-2 cells (2 days old and 9 days old) were incubated in the arginine-free medium ± 1 mmol/L arginine for 3 hours. The cells were then washed three times with the arginine-free medium and reincubated in the arginine-free medium without arginine for another 3 hours. The increased System y^+ activity after 3 hours of arginine incubation diminished, and transport activity returned to prestimulation levels after the cells had been in arginine-free medium for 3 hours (Fig. 6).

System y^+ Activity Increased by Arginine Exposure Was a Kinetic Modification Effect

The System y^+ [^{3}H]-arginine (0.1 μmol/L to 5 mmol/L) uptake kinetics were measured in Caco-2 cells that had been in the arginine-free medium with or without 1 mmol/L arginine (1 mmol/L mannitol as control) for 3 hours. L-Arginine exposure increased both System y^+ transport affinity K_m (30 ± 4 μmole/L vs. 81 ± 6 μmole/L control, n = 9, $P < 0.01$), and maximal capacity V_{max} (2.75 ± 0.2 nmole/mg protein/min vs. 0.25 ± 0.02 nmole/mg protein/min control, n = 9, $P < 0.001$) (Fig. 7). Passive diffusion was not affected by the arginine induction (permeability coefficient P = 1.2 ± 0.1 μl/mg protein/min vs. 1.1 ± 0.1 control, n = 9, $P > 0.05$).

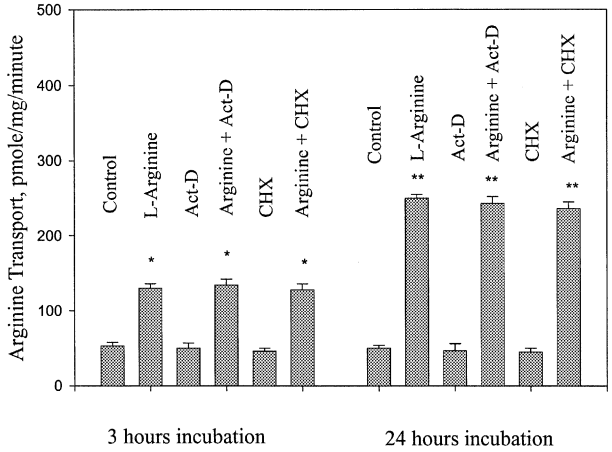


Fig. 5. The effect of actinomycin-D and cycloheximide on the arginine-induced System y^+ activity. Uptake of [^{3}H]-L-arginine (5 μmol/L) was measured in day 2 cells incubated in arginine-free medium containing L-arginine (0 or 1 mmol/L) ± actinomycin-D (Act-D, 0.5 μmol/L) or cycloheximide (CHX, 10 μmol/L) for 3 hours or 24 hours. Transport values are means ± SEM (n = 9). *$P < 0.01$ vs. control; **$P < 0.001$ vs. control.

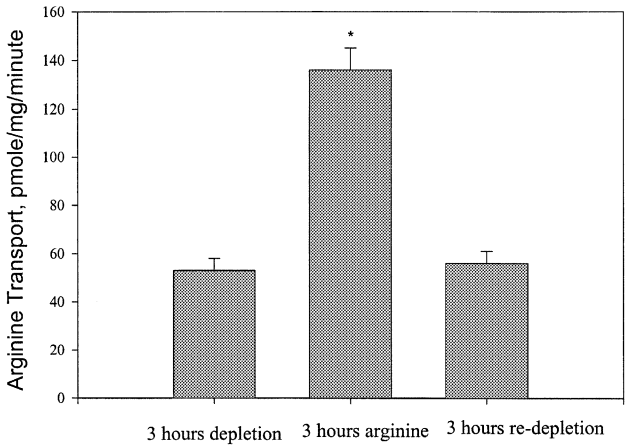


Fig. 6. The arginine-induced System y^+ activity stimulation was reversible. Uptake of [^{3}H]-L-arginine (5 μmol/L) was measured in day 2 cells reincubated in arginine-free medium for 3 hours after having been continuously incubated in arginine (1 mmol/L) for 3 hours previously. Transport values are means ± SEM (n = 9). *$P < 0.01$ vs. control.

System y^+ Activity Increased by Arginine Chronic Exposure Was Not a Protein Synthesis–Dependent Process

Similar to results of the short-term arginine exposure study, the System y^+ activity increased sevenfold with cells incubated in arginine-free medium

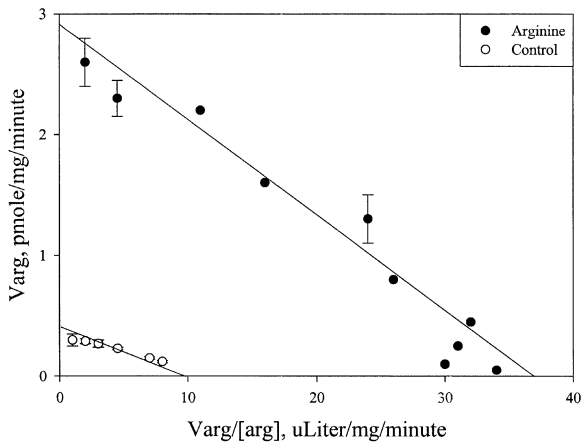


Fig. 7. Eadie-Hofstee plot of System y^+ arginine uptake kinetics. System y^+ arginine (0.1 μmol/L to 10 mmol/L) uptake was measured in day 2 cells incubated in arginine-free medium ± arginine (1 mmol/L) for 3 hours. Kinetic analysis indicated that L-arginine increased System y^+ K_m from 30 ± 4 μmole in the control group and 81 ± 6 μmole in the arginine group (n = 9, $P < 0.01$) and V_{max} from 2.75 ± 0.2 nmole/mg protein/min in the control group to 0.25 ± 0.02 nmole/mg protein/min in the arginine group (n = 9, $P < 0.01$).

plus arginine for 24 hours. This arginine-induced System y^+ activity returned to control levels after the cells had been reincubated in amino acid arginine–free medium for 3 hours.

Neither cycloheximide (10 μmol/L) nor the specific protein kinase C inhibitor chelerythrine chloride (6.6 μmol/L) in the arginine-free medium had any effect on the sevenfold increase in System y^+ activity after 24 hours of arginine exposure (Fig. 8).

DISCUSSION

L-Arginine is a semiessential amino acid in a normal environment, and it is an essential amino acid in weaning cats and dogs, and in trauma, where demand is high.[30–33] Exogenous arginine is needed in the preceding conditions. It must come from either dietary sources or by intravenous means. In its relation to immunity, it is interesting that one of the genes encoding System y^+, CAT-1, has been determined to be the same gene that encodes for a receptor of murine leukemia virus in mouse cells,[34,35] and System y^+ substrates may participate in blocking entry of murine retroviruses. Other animal studies have demonstrated that oral arginine supplementation improves T-cell function and inhibits sarcoma growth and improves animal survival.[36,37] In septic animal studies, dietary arginine has been shown to improve immune function, decrease bacterial translocation, and reduce mortality under different insults in numerous animal studies. Dietary arginine supplementation enhances natural killers and lymphokine-activated killer cell cytotoxicity.[36] Improved survival and lower bacterial translocation events were demonstrated in mice that were fed arginine to reduce sustained intra-abdominal sepsis.[38–42] Similarly, survival of burned pigs also increased with arginine supplementation.[43] Recent clinical trials on trauma, ICU, and cancer patients have demonstrated that enteral feeding supplemented with arginine improves gut function and reduces septic complication and hospital costs.[5–15] But the mechanism by which dietary arginine modulates nutrient absorption is still unknown.

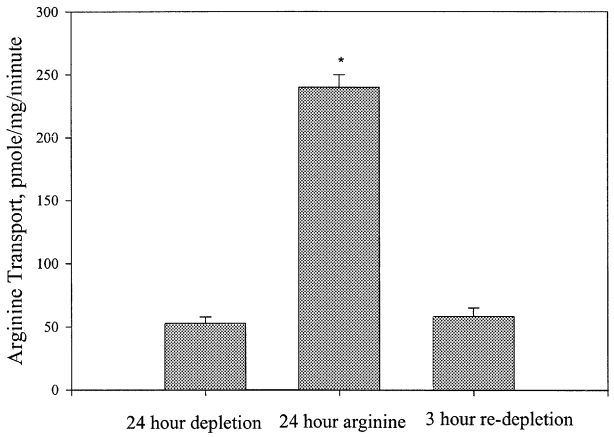


Fig. 8. Chronic arginine-induced System y^+ activity stimulation induction was reversible. Caco-2 cells were incubated in arginine-free medium and arginine-free medium plus arginine (1 mmol/L) for 24 hours. Arginine (5 μmol/L) uptake was measured after these day 2 cells had been reincubated in arginine-free medium for 24 hours. Transport values are means ± SEM (n = 9). $^*P < 0.01$ vs. control.

In previous studies with Caco-2 intestinal epithelial cells, we have characterized System y^+ arginine transport and investigated the regulation of System y^+ arginine transport activity by cell differentiation states and epidermal growth factors.[9,44] In the present study we explored how availability of System y^+ substrates and other amino acids can regulate System y^+ arginine transport activity in a well-defined Caco-2 cell culture.

Intestinal epithelia are exposed to, and adapt to, varying amino acid concentrations in the luminal dietary composition. Animal studies have demonstrated that the small intestine can selectively increase the ability to transport a given nutrient, in response to augmented loads of specific dietary nutrients, such as amino acids, carbohydrates, and vitamins.[18–24] This adaptive upregulation within the small intestine is in stark contrast to cells of internal organs, whereby downregulation of amino acid transport capability occurs in response to specific substrates[16,17] The advantage of this adaptation within the intestine is the maintenance of an adequate absorption capacity that permits the organisms to efficiently keep up with dietary intake. One such mechanism involved in the upregulation of transport activity is termed "transmembrane stimulation." This concerns the kinetic activation of enterocyte transporters already expressed in the membrane. The membrane transporters provide the flexibility for maximum nutrient extraction at any given time.[45]

In the present study we explored the substrate regulation of System y^+ arginine transport across apical membrane in an intestinal epithelial cell model. As shown in Fig. 1, absence of extracellular amino acids downregulated the System y^+ arginine transport activity to a baseline level. The decrease in transport occurs in a relatively rapid pattern, a process that can be explained by the decrease in the intracellular arginine concentration secondary to the metabolism of arginine in the enterocyte. The decreased activity reached its lowest level at approximately 3 hours in an amino acid–free medium and lasted for at least 48 hours. The data suggest that arginine is quickly metabolized in the absence of extracellular arginine. In vivo intestinal studies with intact

intestines have shown that the minimal transport capacity is determined by genetic hard wiring or by the needs of the cell.[17,18,45]

The exogenous arginine-induced System y^+ arginine transport occurred as early as 5 minutes in amino acid–depleted cells. Such short-time action precludes the possibility of transcriptional or translational regulation involvement, a process that requires at least 30 minutes to several hours. Insensitivity of this acute phase of System y^+ stimulation to cycloheximide further rules out the mechanism of new transport protein synthesis.

The System y^+ activity was induced by exogenous amino acids in such a pattern that the System y^+ substrates were the strongest System y^+ stimulators, the weaker System y^+ substrates were also weaker stimulators, and non–System y^+ substrates did not stimulate the System y^+ activity at all. This pattern strongly indicates that the specific System y^+ substrates selectively stimulated the System y^+ arginine uptake (see Fig. 6).

There are two activation mechanisms that could account for the acute-phase stimulation observed in our study: (1) substrate trans-stimulation and (2) translocation/mobilization of existing but latent transporters. Our kinetic analyses revealed that both the System y^+ maximal capacity V_{max} and transport affinity K_m were modulated by exposure to arginine. Translocation kinetics would show only increased transport maximal capacity V_{max} with little or no change in K_m. Taken together with the lack of inhibition by protein kinase C agents (see Results), the data suggest that the increase in System y^+ activity is largely due to trans-stimulation rather than translocation/mobilization of transporter proteins. Western blot analysis using the specific System y^+ transporter MCAT–1 antibody (not commercially available) would further clarify the location of System y^+ transporter protein in individual cellular subcompartments. That passive diffusion coefficient of arginine transport was not affected by arginine induction suggests that this arginine-induced arginine absorption activation is not due to a passive gradient effect.

Similar to in vivo animal studies, prolonged exposure of Caco-2 cells to arginine did not stimulate synthesis of new transporters, as indicated by insensitivity to cycloheximide. Similar to the acute-phase stimulation, the increase in System y^+ activity by prolonged arginine incubation diminished to baseline control values shortly after incubation was discontinued. This reversibility suggested that prolonged and acute incubation shared the same trans-stimulation mechanism.

Taken with data from in vivo intestinal absorption studies, our results suggest that adaptation to dietary essential amino acids is biphasic. Amino acid exposure in first-generation enterocytes of our Caco-2 model simulates the exposure of dietary nutrients to the single population of epithelial cells that arise from crypt stem cells in the intact epithelium. In these cells, uptake is apparently upregulated by trans-stimulation of existing transporters with the resulting enhanced nutrient extraction of a meal. In the intact organism, initial dietary exposure to a given class of substrate would invoke this acute trans-stimulation mechanism, thereby allowing the organisms to rapidly accommodate its new exposure to a substrate-rich diet. In the second phase of the response, the potential for toxicity overaccumulation of certain essential amino acids, such as lysine and arginine, is addressed by the intestine in subsequent generations of enterocytes.[14,45] These differentiated cells arise from the crypt and accumulate on the absorptive villus surface during prolonged (days or weeks) exposure to the substrates.[18,19,24] Thus the intact epithelium in vivo can prevent the ensuing generations of enterocytes—that is, those that arise from the crypt—from overexpressing the appropriate transporter proteins. In addition to the present study, which points to acute-phase trans-stimulation in enterocytes, ongoing studies in our laboratory are addressing the prolonged aspect of the biphasic nature of nutrient adaptation.

REFERENCES

1. Barbul A. Arginine and immune function. Nutrition 1990;6: 53–58.
2. Visek WJ. Arginine needs, physiological state and usual diets. J Nutr 1986;116:36–46.
3. Jeevanandam M, Begay CK, Holaday NJ, Petersen SR. Nutritional and metabolic effects and significance of mild orotic aciduria during dietary supplementation with arginine or its organic salts after trauma injury in rats. Metabolism 1997;46:785–792.
4. Blachier F, Darcy-Vrillon B, Sener A, Duee P, Malaisse WJ. Arginine metabolism in rat enterocytes. Biochim Biophys Acta 1991;1092:304–310.
5. Daly JM, Lieberman MD, Goldfine J, Shou J, Weintraub F, Rosato EF, Lavin P. Enteral nutrition with supplemental arginine, RNA, and omega-3 fatty acids in patients after operation: Immunologic, metabolic, and clinical outcome. Surgery 1992;112:56–67.
6. Daly JM, Weintraub FN, Shou J, Rosato EF, Lucia M. Enteral nutrition during multimodality therapy in upper gastrointestinal cancer patients. Ann Surg 1995;221:327–338.
7. Gianotti L, Braga M, Vignali A, Balzano G, Zerbi A, Bisagni P, Di Carlo V. Effect of route of delivery and formulation of postoperative nutritional support in patients undergoing major operations for malignant neoplasms. Arch Surg 1997;132:1222–1230.
8. Braga M, Gianotti L, Radaelli G, Vignali A, Mari G, Gentilini O, Di Carlo V. Perioperative immunonutrition in patients undergoing cancer surgery: Results of a randomized double-blind phase 3 trial. Arch Surg 1999;134:428–433.
9. Senkal M, Mumme A, Eickhoff U, Geier B, Spath G, Wulfert D, Joosten U, Frei A, Kemen M. Early postoperative enteral

immunonutrition: Clinical outcome and cost-comparison analysis in surgical patients. Crit Care Med 1997;25:1489–1496.
10. Senkal M, Zumtobel V, Bauer KH, Marpe B, Wolfram G, Frei A, Eickhoff U, Kemen M. Outcome and cost-effectiveness of perioperative enteral immunonutrition in patients undergoing elective upper gastrointestinal tract surgery: A prospective randomized study. Arch Surg 1999;134:1309–1316.
11. Moore FA, Moore EE, Kudsk KA, Brown RO, Bower RH, Koruda MJ, Baker CC, Barbul A. Clinical benefits of an immune-enhancing diet for early postinjury enteral feeding. J Trauma 1995;37:607–615.
12. Kudsk KA, Minard G, Croce MA, Brown RO, Lowrey TS, Pritchard FE, Dickerson RN, Fabian TC. A randomized trial of isonitrogenous enteral diets after severe trauma: An immune-enhancing diet reduces septic complications. Ann Surg 1996;224:531–541.
13. Weimann A, Bastian L, Bischoff WE, Grotz M, Hansel M, Lotz J, Trautwein C, Tusch G, Schlitt HJ, Regel G. Influence of arginine, omega-3 fatty acids and nucleotide-supplemented enteral support on systemic inflammatory response syndrome and multiple organ failure in patients after severe trauma. Nutrition 1998;14:165–172.
14. Bower RH, Cerra FB, Bershadsky B, Licari JJ, Hoyt DB, Jensen GL, Van Buren CT, Rothkopf MM, Daly JM, Adelsberg BR. Early enteral administration of a formula (Impact®) supplemented with arginine, nucleotides, and fish oil in intensive care unit patients: Results of a multicenter, prospective, randomized, clinical trial. Crit Care Med 1995;23:436–449.
15. Galban C, Montejo JC, Mesejo A, Marco P, Celaya S, Sanchez-Segura JM, Farre M, Bryg DJ. An immune-enhancing enteral diet reduces mortality rate and episodes of bacteremia in septic intensive care unit patients. Crit Care Med 2000;28:643–648.
16. Kilberg MS, Stevens BR, Novak DA. Recent advances in mammalian amino acid transport. Annu Rev Nutr 1993;13: 137–165.
17. Stevens BR. Vertebrate intestine apical membrane mechanisms of organic nutrient transport. Am J Physiol. 1992; 263:458–463.
18. Ferraris RP, Diamond JM. Specific regulation of intestinal nutrient transporters by their dietary substrates. Annu Rev Physiol 1989;51:125–141.
19. Ferraris RP, Kwan WW, Diamond J. Regulatory signals for intestinal amino acid transporters and peptidases. Am J Physiol 1988;255:151–157.
20. Ferraris RP, Diamond J, Kwan WW. Dietary regulation of intestinal transport of the dipeptide carnosine. Am J Physiol 1988;25:143–150.
21. Diamond JM, Karasov WH. Adaptive regulation of intestinal nutrient transporters. Proc Natl Acad Sci U S A 1987;84:2242–2245.
22. Laganiere S, Maestracci D, Berteloot A. Adaptation of intestinal sugar and amino acid transport to gastric hyperalimentation. Clin Invest Med 1986;9:176–185.
23. Buddington RK, Chen JW, Diamond JM. Dietary regulation of intestinal brush-border sugar and amino acid transport in carnivores. Am J Physiol 1991;261:793–801.
24. Stein ED, Chang SD, Diamond JM. Comparison of different dietary amino acids as inducers of intestinal amino acid transport. Am J Physiol 1987; 52:626–635.
25. Hidalgo IJ, Raub TJ, Borchardt RT. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 1989;96:736–749.
26. Mohrmann I, Mohrmann M, Biber J, Murer H. Sodium-dependent transport of Pi by an established intestinal epithelial cell line (Caco-2). Am J Physiol 1986;250:323–330.
27. Blais A, Bissonnette P, Berteloot A. Common charactristics for Na+-dependent sugar transport in Caco-2 cells and human fetal colon. J Membr Biol 1987;99:113–125.
28. Brandsch M, Miyamoto Y, Ganapathy Y, Leibach FH. Expression and protein kinase C-dependent regulation of peptide/H+ co-transport system in the Caco-2 human colon carcinoma cell line. Biochem J 1994;299:253–260.
29. Pan M, Malandro M, Stevens BR. Regulation of system y+ arginine transport capacity in differentiating human intestinal Caco-2 cells. Am J Physiol 1995;268(Part 1): 578–585.
30. Barbul A. Arginine: Biochemistry, physiology, and therapeutic implication. JPEN 1986;10:227–238.
31. Morris JG, Rogers QR. Ammonia intoxication in the near-adult cat as a result of dietary deficiency of arginine. Science 1978;199:431–432.
32. Morris JG, Rogers QR. Arginine: An essential amino acids for the cat. J. Nutr 1978;108:1944–1953.
33. Andria G, Sebastio G, Sartorio R. Arginine supplementation in lysinuric protein intolerance. J Inherited Metab Dis 1981;5:61–62.
34. Kim JW, Closs EL, Albritton LM, Cunningham JM. Transport of cationic amino acids by the ecotropic retrovirus receptor. Nature 1991;352:725–728.
35. Wang H, Kavanaugh M, North R, Kabat D. Cell-surface receptor for ecotropic murine retroviruses is a basic amino acid transporter. Nature 1991;352:729–731.
36. Reynolds JV, Daly JM, Shou J, Sigal R, Ziegler MM, Naji A. Immunologic effects of arginine supplementation in tumor-bearing and non-tumor-bearing hosts. Ann Surg 1990;211:202–210.
37. Lieberman MD, Nishioka K, Redmond HP, Daly JM. Enhancement of interleukin-2 immunotherapy with L-arginine. Ann Surg 1992; 215:157–165.
38. Gurbuz AT, Kunzelman J, Ratzer EE. Supplemental dietary arginine accelerates intestinal mucosal regeneration and enhances bacterial clearance following radiation enteritis in rats. J Surg Res 1998;74:149–154.
39. Ersin S, Tuncyurek P, Esassolak M, Alkanat M, Buke C, Yilmaz M, Telefoncu A, Kose T. The prophylactic and theapeutic effects of glutamine- and arginine-enriched diets on radiation-induced enteritis in rats. J Surg Res 2000;89:121–125.
40. Gianotti, Alexander JW, Pyles T, Fukushima R. Arginine-supplemented diet improve survival in gut-derived sepsis and peritonitis by modulating bacterial clearance. The role of nitric oxide. Ann Surg 1993;217:644–653.
41. Adjei AA, Yamauchi K, Nakasone Y, Konishi M, Yamamoto S. Arginine-supplemented diets inhibit endotoxin-induced bacterial translocation in mice. Nutrition 11:371–374.
42. Gennari R, Alexander JW, Eaves-Pyles T. Effect of different combination of dietary additives on bacterial translocation and survival in gut-derived sepsis. JPEN J Parenter Enteral Nutr 1995;19:319–325.
43. Saito H, Trocki O, Liang SL. Metabolic and immune effects of supplemental arginine after burn. Arch Surg 1987;122:784–789.
44. Pan M, Stevens BR. Protein kinase C-dependent regulation of L-arginine transport activity in Caco-2 intestinal cells. Biochim Biophys Acta 1995;1239:27–32.
45. Stevens BR. Amino acid transport in intestine. In Kilberg MS, Häussinger D, eds. Mammalian Amino Acid Transport Mechanisms and Control. New York: Plenum Press, 1992, pp 149–163.

Luminal Regulation of Na^+/H^+ Exchanger Gene Expression in Rat Ileal Mucosa

Marc A. Doble, B.S., Vicky B. Tola, M.D., Stephanie A. Chamberlain, M.D., Robert R. Cima, M.D., Alfred Van Hoek, Ph.D., David I. Soybel, M.D.

It is well recognized that ileostomy patients suffer from chronic depletion of Na^+ through the stoma effluent. In this study we evaluated the effects of ileostomy on messenger RNA levels that encode different Na^+/H^+ exchanger isoforms (NHE-2 and NHE-3). Loop ileostomies were created in Sprague-Dawley rats. Segments of diverted ileum were harvested for quantitation of mRNA levels encoding these isoforms and the Na^+/K^+ ATPase in mucosal scrapings and for immunofluorescence microscopy, specifically of the NHE-3 protein. Our studies indicate that as early as 8 days after diversion, NHE-3 gene expression is selectively attenuated in poststomal ileal mucosa. Mucosal morphology remains undisturbed, and the distribution of protein expression along the crypt/villus axis is not altered. Infusion of Na^+ or the enterocyte nutrient, glutamine, into the lumen of the diverted segment restores or even augments mRNA levels for NHE-3, again without altering the histologic appearance or distribution of the protein along the crypt/villus axis. These effects are specific because nonpolar osmolytes (mannitol) and related organic nutrients not specific for the enterocyte (i.e., butyrate) have no effect on mRNA levels of NHE-3. Further work is required to understand how the early changes in mRNA contribute to mucosal function and response to luminal diversion. (J GASTROINTEST SURG 2002;6:387–395.)

KEY WORDS: Intestinal mucosa, ion transport, Na^+, H^+, glutamine

It is well recognized that ileostomy patients suffer from chronic, systemic depletion of Na^+. In normal individuals, total daily losses in stool are rarely more than 5 to 10 mEq Na^+.[1–3] Among patients who seem to have adapted to the presence of an ileostomy, the average patient loses between 600 and 640 ml of fluid and approximately 70 to 80 mmol of Na^+ through the ileostomy effluent.[2,3] In such patients, systemic Na^+ depletion is associated with a 30% decrease in urinary excretion of Na^+ and a 30% increase in urinary excretion of K^+.[4–6] We hypothesize that early and late rehabilitation of the ileostomy patient will depend on enhancement of upstream mechanisms of Na^+ absorption.

Progress in identifying the mechanisms of intestinal adaptation after ileal diversion has been hindered, in part, because of the absence of well-characterized models of ileostomy in the experimental setting. In preliminary studies, our laboratory has developed an in vivo model of loop ileostomy, without colon resection, in the rat.[7,8] In this study we evaluated the effects of ileostomy on messenger RNA levels that encode different Na^+ transporters, notably the Na^+/H^+ exchanger isoforms NHE-2 and NHE-3, which appear to play a role in normal intestinal and colonic absorption of Na^+ from the lumen.[8–11] These studies included protocols to evaluate whether absorption of luminal Na^+ is a factor that regulates expression of NHE-2 and NHE-3 in mucosa of diverted ileal segments. Our studies indicate that, at the transcriptional level, expression of the NHE-3 isoform is sensitive to ileal diversion and specifically to the presence of Na^+ and organic nutrients in the lumen.

MATERIAL AND METHODS

Animal Model

The study protocol was approved by the Harvard Medical Area Standing Committee on Animals at Harvard Medical School, Boston, Massachusetts, and the Animal Studies Committee of the Boston

Present at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract in Atlanta, Georgia, May 20–23, 2001 (poster presentation).

From the Department of Surgery (M.A.D., V.B.T., S.A.C., R.R.C., D.I.S.), Brigham and Women's Hospital, VA Boston Healthcare System; and the Renal Unit (A.V.H.), Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts.

Supported by grant RO1 DK 44571-11 from the National Institutes of Health and by funds from the Brigham Surgical Group Foundation.

Reprint requests: David I. Soybel, M.D., Division of General and Gastrointestinal Surgery, Brigham and Women's Hospital, 75 Francis St., Boston, MA 02115. e-mail: dsoybel@partners.org

1091-255X/02/$—see front matter
PII: S1091-255X(01)00049-X

VA Healthcare System. Operative procedures were performed in adult, 4- to 6-month-old, male, Sprague-Dawley rats. Animals randomly underwent a sham laparotomy or creation of a loop ileostomy modified from the procedure described previously.[7,8] All animals underwent similar preoperative and postoperative care. This entailed food deprivation 24 hours before and 24 hours after surgery. Before surgery, all animals had access to water. After surgery, animals had ad libitum access to water and 0.25 normal saline solution. Animals were anesthetized by means of a combination of ketamine (40 mg/kg) and xylazine (5 mg/kg) administered intramuscularly. After induction of anesthesia, a 22-gauge, 1-inch intravenous catheter was placed in the tail vein, and a syringe was attached to provide intravenous fluid during the operative and postoperative recovery periods. All animals received a total of 3.0 ml in 0.25 ml boluses of normal saline solution before the intravenous cannula was removed. Body temperature was monitored with a rectal thermistor. During all operations, the body temperature was maintained above 35° C by use of a warming blanket. Postoperative pain control was achieved by subcutaneous injection of Buprenex (buprenorphine HCl, 0.1 mg/kg) administered 4 hours after the animals were fully recovered from the anesthetic and then again 12 hours after that injection. Postoperatively, animal weights were recorded daily. Ileostomy animals were given daily baths on each postoperative day to prevent skin injury from the caustic ileostomy effluent.

All operations were performed under sterile conditions. After the induction of anesthesia, the abdomen was shaved and cleaned with 95% ethanol. In both sham and ileostomy animals, the abdomen was entered through a 4 cm midline incision. In the sham laparotomy animals (N = 11), once the abdomen was entered, the distal small intestine and cecum were identified. The distal small intestine and cecum were then brought out of the midline incision and draped with a warm saline-soaked gauze. Ten minutes after exteriorization, the distal small intestine and cecum were returned to the abdominal cavity. The midline incision was then closed with a running 4-0 vicryl suture. The skin edges were approximated and closed with animal skin staples. In ileostomy animals (N = 9), after the abdomen was entered, a 4 to 6 mm circular stoma was created in the abdominal musculature and skin approximately 1.0 cm to the right of the midline incision. A loop of distal ileum 5.0 cm proximal to the ileocecal valve was delivered through the stoma. The loop of ileum was opened along the antimesenteric border for a distance of 1.0 cm. The free edges of this loop were attached to the abdominal musculature and skin with interrupted 4-0 silk sutures. Before the abdomen was closed, both afferent and efferent limbs of the loop ileostomy were examined for patency by infusion of warm saline solution through a 22-gauge intravenous catheter. The abdominal cavity was closed as previously described.

Tissue Preparation for mRNA

On postoperative day 8 or 21, animals were killed by a pentobarbitol overdose (100 mg/kg). Segments of bowel, 5 cm in length, were harvested and the mucosa was separated rapidly from the underlying muscularis by sharp dissection. Corresponding segments of intestine were harvested from the sham rats. The mucosal tissue was frozen and stored in liquid nitrogen until processed for mRNA. Messenger RNA from the samples was isolated by RNAzol B (Tel-Test Inc., Friendswood, Texas) modified as described previously.[7,8]

Northern Blot Analysis

As described previously,[7,8] total RNA (10:g per lane) was run on a 1% agarose gel containing 0.63% formaldehyde staining of 18 S and 28 S ribosomal RNA. The RNA was then transferred to nylon membranes and underwent ultraviolet cross-linking, washing, and hybridization as described,[8] with 10^6 counts $\times$ min^{-1} $\times$ ml^{-1} ^{32}P-labeled cDNA probe for NHE-2 (2.1 kb pairs), NHE-3 (2.2 kb pairs), and the alpha subunit of the ouabain-sensitive Na/K ATPase (3.6 kb pairs) provided by Eric Delpire, Ph.D. (Vanderbilt University).

The membranes were washed twice at low stringency (room temperature, 300 mmol/L NaCl) and once at higher stringency (65° C, 30 mmol/L NaCl). The membranes were then exposed to x-ray film (Reflexion NEF; DuPont, Boston, Massachusetts) at −80° C for 1 to 3 days. Quantification of relative mRNA abundance was performed via scanning densitometry (Microtek Image Scanner, Microtek International, Inc., Hsinchu, Taiwan; Image 1.49 software from the National Institutes of Health, Bethesda, Maryland). Membranes were allowed several weeks between probing so as to not require stripping. All membranes were probed with ^{32}P-labeled cDNA encoding the housekeeping enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), to provide additional confirmation of the equivalence of gel loading.[7,8]

Histologic Evaluation and Immunofluorescence Studies

In ileostomy and control animals, tissue samples were obtained from the poststomal segment of ileum.

Tissues were washed and flushed intraluminally with 1× phosphate-buffered saline (PBS) solution before fixation in 4% paraformaldehyde solution for 1 hour, then rinsed and stored in PBS–10 mmol/L sodium phosphate buffer containing 0.9% NaCl, pH 7.4) at 4° C until embedding in paraffin and sectioning. For standard histologic examination of tissues, serial thick sections were cut and stained with hematoxylin-eosin. Photomicrographs were obtained at ×250 magnification.

For immunofluorescence labeling, tissues were cryoprotected in 30% sucrose before sectioning with a Reichert Frigocut (Reichert-Jung, Leica, Benheim, Germany) microtome using disposable knives. Thin tissue sections (5 μm) were cut and picked up on Fisher Superfrost (Fischer Scientific, Hanover Park, Illinois) plus slides, then stored at −20° C for at least 24 hours before use for incubations. Slides were rehydrated in 1 × PBS for 5 minutes before blocking in 1% bovine serum albumin solution for 1 hour. Incubation with primary antibody took place for 1 hour at room temperature. After two washings (5 minutes) with high-salt PBS (PBS containing 2.7% NaCl), sections were exposed to primary goat–anti-rabbit antibody coupled to CY3 for the polyclonal primary antibody and then to secondary goat–anti-mouse antibody coupled to fluorescein isothiocyanate (FITC) for 1 hour at room temperature. After additional washings as above, sections were mounted in Vectashield (Vector Labs, Burlingame, Calif.) antifading solution diluted in 1:1 0.1 mol/L Tris:HCl, pH 8.0, a pH at which FITC fluorescence is not quenched. For these studies the following antibodies were obtained: NHE-3 monoclonal antibody was raised against the maltose-binding protein fusion protein containing c-terminal 131 aa of rabbit NHE-3 (Chemicon International, Temecula, California). Secondary antibody applied was an FITC-labeled goat–antimouse (GAM) IgG antibody (Kirkegaards and Perry, Gaithersberg, Maryland).

Sections were photographed in black and white on Kodak Tmax 400 film push-processed to 1600 ASA and in color on Kodak Ektachrome 400 Elite (Kodak, Rochester, New York) film at 1600 ASA. Images were captured directly from the Nikon FXA (Nikon USA, Melville, New York) photomicroscope using an Optronics three-bit CCD color camera, and IP Lab Spectrum (Signal Analytics Corp., Fairfax, Virginia) acquisition and analysis software running on a Power PC 8500 (Apple Computer, Inc., Cupertino, California).

Experimental Protocols

Protocol 1. Adult Sprague-Dawley rats underwent sham laparotomy (N = 11) or loop ileostomy (N = 9), with 5 cm of terminal ileum diverted. Rats were killed 8 days after surgery. Another similar group of rats (N = 6 each) was killed 21 days after surgery and mucosa from the diverted 5 cm segment of ileum was harvested for analysis. Total RNA was isolated from mucosal scrapings of several regions of the small intestine in ileostomy animals and in animals that had undergone sham operation. These included the following: a 5 cm segment of jejunun taken just distal to the duodenum; a 5 cm segment of mid–small bowel taken midway between the duodenum and the ileocecal valve; the 5 cm segment of ileum just proximal to the stoma; and the 5 cm segment just distal to the stoma. Northern blot analysis was used to measure mRNA levels for Na^+/K^+ ATPase (a housekeeping transporter that maintains intracellular Na^+ levels) and the NHE isoforms NHE-2 and NHE-3.

Protocol 2. To determine whether luminal Na^+ might influence NHE expression, three groups of ileostomy rats (N = 5 each) were prepared as follows: a control group that received no luminal infusion; two other groups that received twice-daily infusions of 3 ml solutions containing either normal saline (308 mOsm) or mannitol (308 mOsm) into the lumen of the diverted ileum. Infusions were performed into the distal limb of the ileostomy, in prograde fashion, over a period of 3 to 5 minutes. Rats were killed 8 days after ileostomy and tissues were harvested for analysis.

Protocol 3. In the last group of studies, groups of rats (N = 5 each) received twice-daily infusions of solutions containing glutamine (25 mol/L in sterile normal saline) or butyrate (25 mol/L in sterile normal saline). Rats were killed 8 days after ileostomy and tissues were harvested for analysis.

Statistical Analysis

Data were recorded and analyzed with a standard software statistical package (Excel, Microsoft Corp., Redmond, Wash.). Where appropriate, comparisons of measurements between experimental groups underwent analysis of variance (ANOVA) for multiple measurements.

RESULTS

Animal Responses to Ileostomy

Animal weights were monitored daily throughout the study. Before the procedures, there were no significant differences among the groups. As reported previously,[8] sham animals initially lost weight but regained weight by the end of the first postoperative week (data not shown). All animals undergoing ileostomy, however, lost weight to varying degress. Ileostomy animals

had severe postoperative diarrhea from postoperative day 2 to approximately day 8. In general, ileostomy animals lost 30% of their body weight ($P < 0.001$) compared to preoperative weight (by paired *t* test) but maintained normal, healthy behavior until they were killed.

Gross Mucosal Appearance

At the time of death, the stomach and intestines of each animal were inspected. In sham animals, the stomach typically appeared to be full of solid food. Variable amounts of succus were visible in the small intestine. The cecum and colon were invariably full of semisolid fecal matter. In the ileostomy group, the small intestine proximal to the stoma was mildly dilated. The ileum distal to the stoma, the cecum, and the colon were devoid of luminal contents, thus confirming total diversion of the fecal stream. In the ileostomy group, just before harvest of mucosal scrapings, inspection revealed dilatation of the lumen along all regions proximal to the stoma. At 8 days after surgery, it was difficult to discern atrophy or hypertrophy of the mucosa in the diverted regions, compared to sham animals. Distal to the ileostomy, the lumen of the ileum was contracted, but the mucosa itself was not noticeably atrophied, compared to sham animals.[12]

Histologic Findings

Histologic sections, stained with hematoxylin-eosin, were obtained from the different regions of the small intestine in three sham-operated and three ileostomy animals. As noted previously,[12] there was little visible difference between ileum of sham-operated animals compared to the animals that had undergone diversion (data not shown). In the segments of ileum proximal and distal to the stoma, there were no observable differences in villus/crypt height ratio. There was slight flattening of the villus tips in diverted areas, a slight increase in the number of intraepithelial lymphocytes, and increases in density of goblet cells, compared to the corresponding terminal ileum in control animals. The absence of clear changes in histologic appearance was true for specimens taken from all groups of control and diverted animals, at 8 days and at 21 days after diversion. This was also true for all groups of animals undergoing various infusions into the diverted ileum.

Regional Variations in Levels of mRNA Encoding NHE-2, NHE-3, and Na/K ATPase

Figure 1 summarizes measurements of levels of mRNA encoding different transporters, derived from control rats in the four different regions of the rat small intestine. These included "proximal jejunum" (identified by its proximity to the duodenum), "midjejunum" (identified by its position in the midpoint between the duodenum and cecum), the segment of terminal ileum 5 cm proximal to the stoma ("prestoma"), and the segment of terminal ileum that was diverted ("poststoma"). As shown, levels of mRNA encoding the housekeeping transporter, Na/K, and those of mRNA encoding the NHE-2 isoform did not vary significantly along the length of the small intestine. In contrast, mRNA levels of the NHE-3 isoform were increased significantly in downstream regions of the small intestine.

The figure also summarizes measurements of GAPDH mRNA levels in these same regions of the intestine. This is one of several markers, including actin and ubiquitin, which have been used for evaluating equivalence of gel loading. In the course of these studies we found that, within any given region of the intestine, mRNA levels of GAPDH did not vary significantly under different experimental conditions. At the same time, GAPDH mRNA levels decreased significantly in downstream regions of the intestine. Thus it would be reasonable to use GAPDH mRNA levels as a reference for normalizing results. However, GAPDH mRNA levels cannot be used with confidence if the intention is to compare measurements obtained in different regions of the intestine. In subsequent figures, all measurements of mRNA levels in the diverted ileal segment are summarized after normalization to GAPDH.

Measurements of mRNA Levels Encoding NHE Isoforms in Diverted Segments

Figure 2 summarizes the effects of ileal diversion on mucosal mRNA levels in the poststomal ileum at 8 days and 21 days after diversion. Despite the relatively minimal changes in histologic appearance, decreases in NHE-3 mRNA levels were observed as early as 8 days and most markedly at 21 days after luminal diversion. Little if any change was noted in levels of mRNA encoding for Na/K or the NHE-2 isoform.

Measurements of mRNA Levels Encoding NHE Isoforms During Infusion of Luminal Na^+ or Nutrients Into Diverted Segments

Figure 3 summarizes studies in which small volumes (3 ml) were infused intermittently into the diverted, poststomal ileum. The infused solutions included isotonic saline (159 mmol/L) or isoosmotic (308 mmol/L) quantities of a nonabsorbed nonionic osmolyte, mannitol. Control animals underwent instrumentation of the ileostomy by the catheter, without actual infusion of any solution. As shown, infusion of mannitol had little

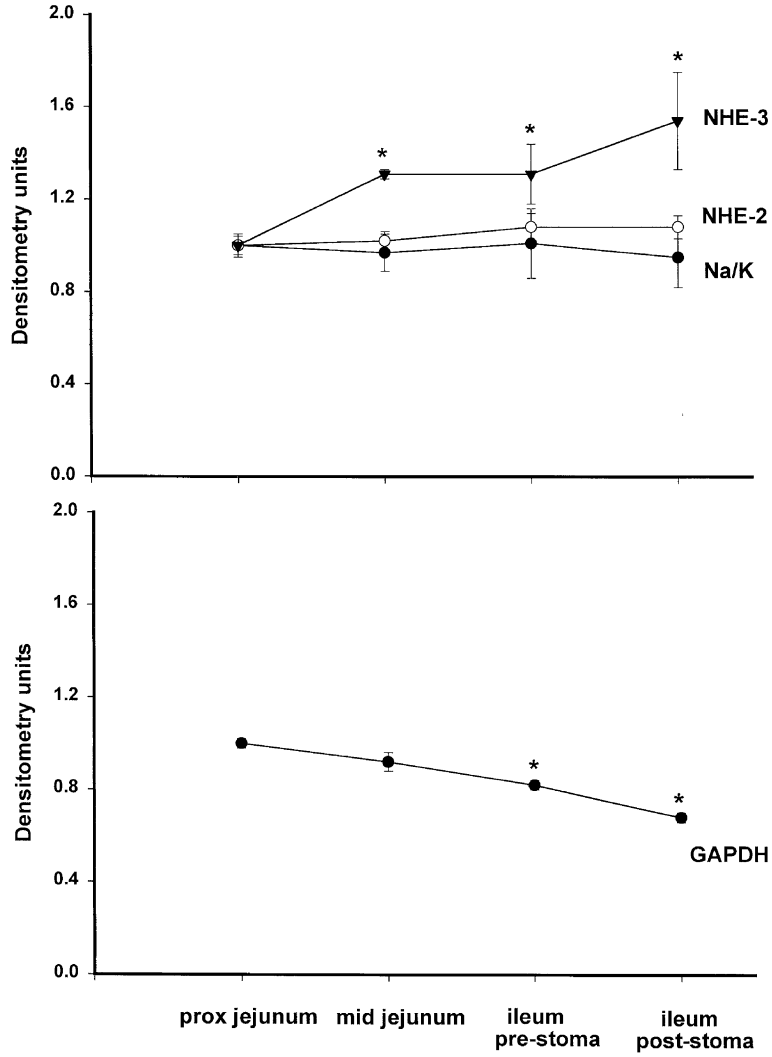


Fig. 1. Messenger RNA content of mucosal scrapings from control animals (N = 11) in different regions of the small intestine. Sites of harvest as indicated in the figure. *$P < 0.05$ compared to mRNA levels in proximal jejunal tissues (ANOVA).

impact on postileostomy levels of mRNA encoding for NHE-3. In contrast, infusion of NaCl had a marked stimulatory effect on NHE-3 gene expression in the diverted segment, averaging 60% above levels seen in animals that had undergone ileostomy without infusion. Again, no significant changes were noted in levels of mRNA encoding for Na/K; significant but quantitatively small decreases were noted in mRNA levels for the NHE-2 isoform.

Figure 4 shows the effects of infusing two organic nutrients into the diverted poststomal ileum: butyrate, a small-chain fatty acid known to stimulate colonocyte growth and differentiation[13,14]; and glutamine, a small similarly sized amino acid well recognized as a nutrient for enterocyte growth and differentiation.[15,16] As shown, butyrate had no effects beyond those of saline on mRNA levels encoding Na/K, NHE-2, or NHE-3. In contrast, infusion of glutamine markedly enhanced NHE-3 mRNA levels, averaging 80% above the levels observed with saline infusion alone. Again, little if any change was noted in levels of mRNA encoding for Na/K or the NHE-2 isoform.

Immunhistochemistry of NHE-3 Expression After Diversion

Immunohistochemical studies were performed using specific antibodies to NHE-3, 8 days after sham operation (N = 6) or ileostomy (N = 5). As shown in Fig. 5, there was no clear alteration in the distribution of NHE-3 expression along the length of the villus. Although qualitative in nature, these figures also suggest that at 8 days there was very little actual loss of the NHE-3 protein or the cells expressing it.

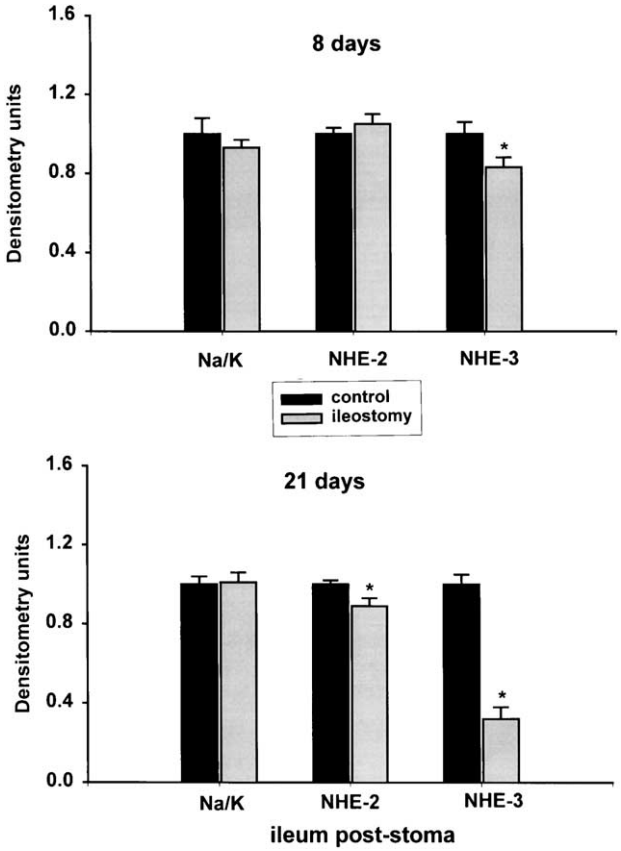


Fig. 2. Messenger RNA content of mucosal scrapings from poststomal ileum harvested from ileostomy animals (N = 9) and from the corresponding segments of ileum in control animals (N = 11). mRNA levels from tissues harvested 8 days after diversion (upper panel). mRNA levels harvested 21 days after diversion (lower panel). *$P > 0.05$ compared to mRNA levels in tissues harvested from control (sham-operated) animals (ANOVA).

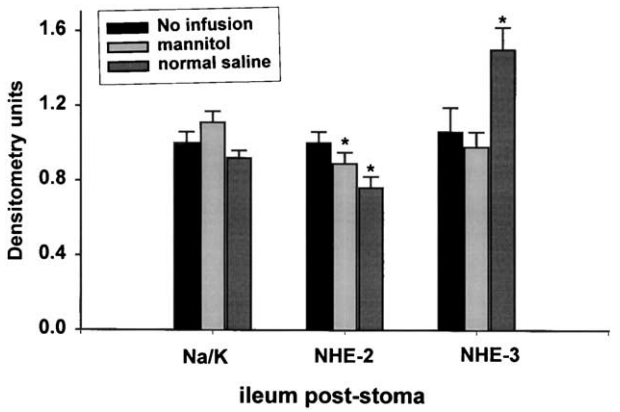


Fig. 3. Messenger RNA content of mucosal scrapings in mucosal scrapings harvested from poststomal segments of ileum with no infusion or with infusions of isoosmotic mannitol or saline. mRNA levels from tissues harvested 8 days after diversion are shown. *$P < 0.05$ compared to mRNA levels in tissues harvested from animals that did not receive infusions (ANOVA).

Similarly, we found that infusion of saline solution did little to alter the distribution of the fluorescence signal (Fig. 6). In those animals receiving infusions of saline plus glutamine (but not butyrate), the signal was uniformly thicker and more intense in the enterocytes located in the upper third of the villus (N = 5 in all groups).

DISCUSSION

In this experimental study, we evaluated the effect of luminal diversion on gene expression of transporters potentially involved in salt and water absorption in the distal ileum. Our goal was to determine whether loss of luminal content alters expression, at the level of mRNA or protein synthesis. In addition, we were interested in determining whether specific luminal factors (such as the Na^+ ion) or small organic nutrients (such as butyrate or glutamine) might be important in maintaining baseline levels of expression. A key part of our experimental strategy was to determine whether organic nutrients would elicit their effects over and above the presence of Na^+ ions, which are always present in luminal contents and endogenous secretions.

Our results indicate that deprivation of luminal contents decreases gene expression of the NHE-3 isoform of the Na^+/H^+ exchanger in the mucosa of the distal ileum. In contrast, mRNA levels for NHE-2 and the Na^+/K^+ ATPase were not significantly altered by diversion. The absence of marked atrophic changes in histologic appearance of diverted mucosa, at 8 and even 21 days after diversion, was somewhat surprising. However, this relative lack of change in mucosal morphology increases confidence that any observed alterations in expression would not simply be due to cell loss. These findings suggest that diversion specifically affects gene expression of the NHE-3 isoform, which is thought to be most important in salt-mediated water absorption in the ileum and colon.[17–19]

Our results also indicate that the presence of certain constituents of the luminal environment, such as Na^+ and glutamine, can reverse decreases or even augment gene expression of NHE-3 in the diverted mucosa. In contrast, no sizeable effects were observed with respect to expression of NHE-2 or the Na^+/K^+ ATPase. A nonmetabolizable osmolyte, mannitol, had no such preservative effect on NHE-3 mRNA content in the mucosa. Neither was such preservation of NHE-3 mRNA levels observed with

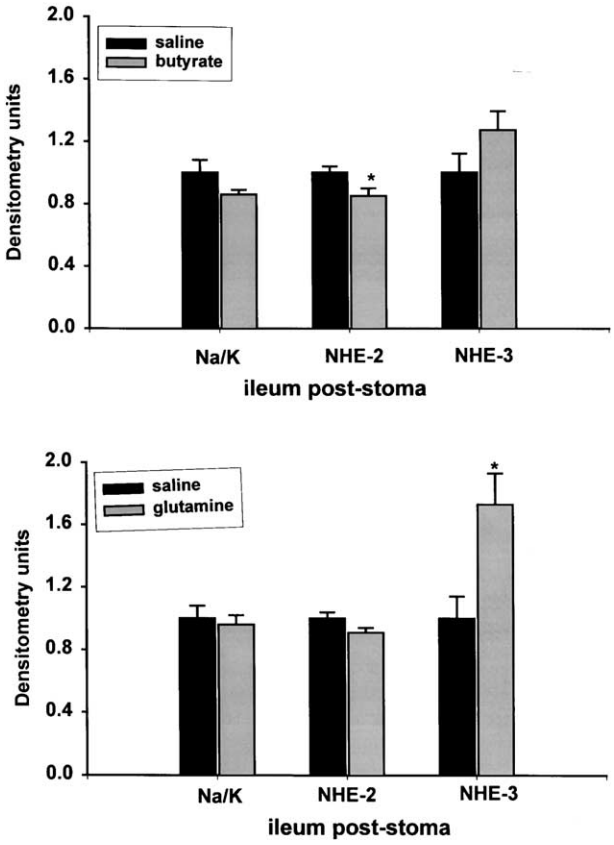


Fig. 4. Messenger RNA content of mucosal scrapings in mucosal scrapings harvested from poststomal segments of ileum with infusion of saline alone vs. saline plus butyrate (*upper panel*); and infusion of saline alone vs. saline plus glutamine (*lower panel*). Panel shows mRNA levels from tissues harvested 8 days after diversion. $^*P < 0.05$ compared to mRNA levels in tissues harvested from animals that received infusions of saline only (ANOVA).

intraluminal administration of butyrate, an organic nutrient structurally related to glutamine and known as a colonocyte nutrient but not as an enterocyte nutrient. Thus it appears that, at the transcriptional level, NHE-3 expression responds to luminal presence or absence of the Na^+ ion and specific enterocyte trophic factors.

Somewhat puzzling was the observation that distribution of the NHE-3 protein was not altered by diversion at 8 days after diversion. To be sure about this finding, similar studies were performed in three animals 21 days after diversion. As was observed only 8 days after diversion, no real changes in distribution of the protein in the brush border and along the crypt/villus axis of the ileal mucosa were seen (data not shown). It is possible that quantitative measurements of protein using Western blot analysis would reveal changes in protein content; however, the qualitative appearance of immunofluorescence intensity (see Figs. 5 and 6) argues against there being profound alterations in NHE-3 protein expression, even with a 3-week interval after diversion. These findings suggest that NHE-3 expression is altered, at the level of gene transcription, soon after diversion. However, expression levels and distribution of the NHE-3 protein product may not be altered in the short term.

In this context, it has become increasingly well recognized that transcription of the message and translation to transport proteins such as NHE-3 are not always correlated.[19,20] Caution must be used in drawing conclusions about the effects of specific manipulations of luminal conditions or the neurohumoral milieu on transporter expression and function. At the same time we do not believe it would be fair to conclude that, because message and protein levels do not always correlate, changes in message level are unimportant in understanding mucosal responses to changing conditions.[21] Our findings suggest that diversion does not immediately downregulate protein expression but may have effects on the mucosal investment in new production of message and protein. At the transcriptional level, expression of NHE-3 is

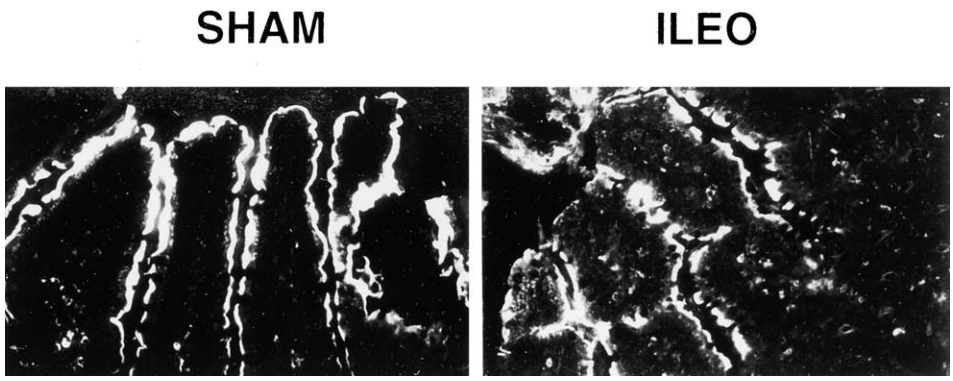


Fig. 5. Immunohistochemistry using NHE-3 antibody in the poststomal ileal segment in a diverted animal (*left*) and from the corresponding 5 cm segment of terminal ileum in a control animal (*right*).

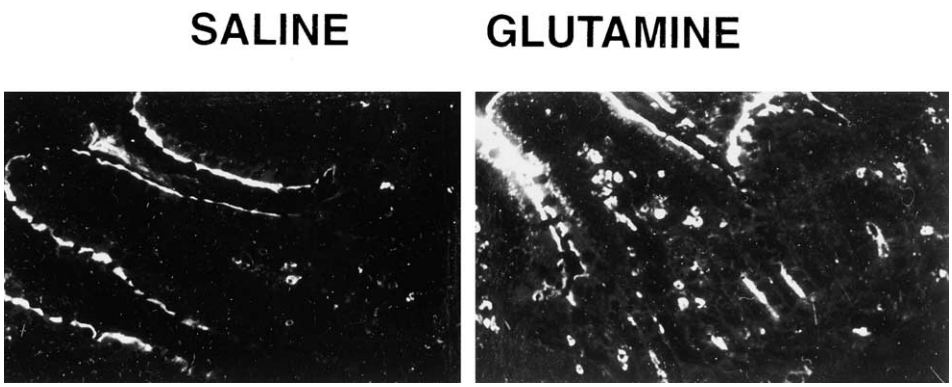


Fig. 6. Immunohistochemistry using NHE-3 antibody. Poststomal ileal segment infused by saline (*left*) vs. ileostomy with infused by saline and glutamine (*right*). Tissues harvested 8 days after operation. (Original magnification ×200.)

sensitive to the ion and nutrient composition of the fluid within the ileal lumen. Further work is required to understand whether and how these early changes in message level contribute to mucosal function and the adaptive response to luminal diversion.

In summary, our studies suggest that content and composition of the lumen may influence transcription and ultimately expression of specific transporters that mediate salt and water transport in the mucosa of the distal ileum. The recognition that expression of NHE-3 may be the most sensitive indicates that this isoform is a potential target of manipulation for improving salt and water absorption. In addition, the fact that alterations in content and composition of the lumen may affect later expression offers the possibility that manipulation of diet or luminal nutrients may prove therapeutically useful in patients suffering from excessively high output from an ileostomy or with diseases affecting absorption in the distal ileum.

REFERENCES

1. Soybel DI. Adaptation to ileostomy. Surgery 2001;129: 123–127.
2. Kelly DG, Branon ME, Phillips SF, Kelly KA. Diarrhoea after continent ileostomy. Gut 1980;21:711–716.
3. Taylor BM, Cranley B, Kelly KA, Phillips SF, Beart RW Jr, Dozois RR. A clinicophysiological comparison of ileal pouch-anal anastomosis and straight ileoanal anastomosis. Ann Surg 1983;198:462–468.
4. Huber F-X, Stern J, Hinz U, Werle E, Haack D, Kienle P, Heuschen U, Aulmann M, Decker-Baumann C, Herfarth C. Effects of restorative proctocolectomy on renal and adrenal function. Dis Colon Rectum 1999;42:1318–1324.
5. Kramer P. The effect of varying sodium loads on the ileal excreta of human ileostomized subjects. J Clin Invest 1966; 45:1710–1718.
6. Kennedy HJ, Al-Dujaili EAS, Edwards CRW, Truelove SC. Water and electrolyte balance in subjects with a permanent ileostomy. Gut 1983;24:702–705.
7. Hallonquist H, Cima RR, Klingensmith ME, Purdy J, Delpire E, Zinner M, Soybel DI. Selective increase in gastric mucosal mRNA encoding basolateral Na-K-2Cl cotransporter following ileostomy in the rat. J GASTROINTEST SURG 1998;2:238–243.
8. Doble MA, Tola VB, Cima RR, Zinner MJ, Klein MA, Soybel DI. Luminal osmolarity downregulates gene expression of Na^+/H^+ exchangers in colonic mucosa. J GASTROINTEST SURG 2000;4:531–535.
9. Bookstein C, DePaoli AM, Xie Y, Niu P, Musch MW, Rao MC, Chang EB. Na/H exchangers, NHE1 and NHE3 of rat intestine. Expression and localization. J Clin Invest 1994;93:106–113.
10. Brant SR, Yun CHC, Donowitz M, Tse CM. Cloning, tissue distribution, and functional analysis of human Na/H exchanger isoform, NHE3. Am J Physiol 1995;269:198–204.
11. Collins JF, Honda T, Knobel S, Bulus NM, Conary J, DuBois R, Ghishan FK. Molecular cloning, sequencing, tissue distribution and functional expression of a Na+/H+ exchanger (NHE-2). Proc Natl Acad Sci U S A 1993;90:3938–3942.
12. Purdy MJ, Cima RR, Doble MA, Klein MA, Zinner MJ, Soybel DI. Selective decreases in levels of mRNA encoding a water channel (AQP3) in ileal mucosa after ileostomy in the rat. J GASTROINEST SURG 1999;3:54–60.
13. Roediger WEW. Utilization of nutrients by isolated epithelial cells of the rat colon. Gastroenterology 1982;83: 424–429.
14. Velazquez OC, Lederer HM, Rombeau JL. Butyrate and colonocyte. Implications for neoplasia. Dig Dis Sci 1996; 41:727–739.
15. McGivan JD, Pastor-Anglada M. Regulatory and molecular aspects of mammalian amino acid transport. Biochem J 1994;299:321–334.
16. Rhoads JM, Argenzio RA, Chen W, Rippe RA, Westwick JK, Cox AD, Berschneider HM, Brenner DA. L-glutamine stimulates intestinal cell proliferation and activates mitogen-activated protein kinases. Am J Physiol 1997;272(Part 1):G943–G953.
17. Hoofgerwerf WA, Tsao SC, Devuyst O, Levine SA, Yun CH, Yip JW, Cohen ME, Wilson PD, Lazenby AJ, Tse CM,

Donowitz M. NHE2 and NHE3 are human and rabbit intestinal brush border proteins. Am J Physiol 1996;270:29–41.
18. Shulteis PJ, Clarke LL, Meneton P, Miller ML, Soleimani M, Gawenis LR, Riddle TM, Duffy JJ, Doetschman T, Wang T, Giebisch G, Aronson PS, Lorenz JN, Schull GE. Renal and intestinal absorptive defects in mice lacking the NHE3 Na^+/H^+ exchanger. Nature Genetics 1998;19:282–285.
19. Maher MM, Gontarek JD, Bess RS, Donowitz M, Yeo CJ. The Na+/H+ exchange isoform NHE3 regulates basal canine ileal absorption in vivo. Gastroenterology 1997;112: 174–183.
20. Collins JF, Kiela PR, Xu H, Ghishan FK. Expression of rat renal NHE2 and NHE3 during post-natal development. Biochim Biophys Acta 2000;1464:7–17.
21. Cho JH, Musch MW, Bookstein CM, McSwine RL, Rabeneau K, Chang EB. Aldosterone stimulates Na+ absorption in rats by increasing NHE3 expression of the proximal colon. Am J Physiol 1998;274:586–594.
22. Yun CH, Gurubhagavatula S, Levine SA, Montgomery JL, Brant SR, Cohen ME, Cragoie EJ Jr, Pouyssegur J, Tse CM, Donowitz M. Glucocorticoid stimulation of ileal Na+ absorptive cell brush border Na+/H+ exchange and association with an increase in message for NHE-3, an epithelial exchanger isoform. J Biol Chem 1993;268:206–211.

Poor Outcome and Quality of Life in Female Patients Undergoing Secondary Surgery for Recurrent Peptic Ulcer Disease

Gonzalo V. Gonzàlez-Stawinski, M.D., Jason M. Rovak, M.S., Hilliard F. Seigler, M.D., John P. Grant, M.D., Matthew F. Kalady, M.D., Shanka Biswas, M.D., Theodore N. Pappas, M.D.

Secondary peptic ulcer surgery is uncommon given the success of a wide variety of medical therapies, plus the good outcome expected after primary peptic ulcer surgery. Early reports of secondary peptic ulcer surgery in the 1950s and 1960s suggested good long-term outcome in most patients; however, recent data suggest that patients operated in the *Helicobacter pylori* era have a worse outcome. We have attempted to quantify the poor outcome in these patients and measure the effect of sex, a previously unrecognized risk factor for poor outcome after peptic ulcer surgery. We reviewed the outcomes of 35 patients who underwent secondary peptic ulcer surgery for symptoms of persistent or recurrent peptic ulcer symptoms or complications of the condition. These patients were compared to a "control" group of patients to determine long-term quality of life as measured by the SF-36 and Visick scores (average follow-up 60 months). Visick and SF-36 scores were obtained through telephone interviews. The two groups of patients were age matched to eliminate age as a variable in the SF-36 results. There were more females than males in the secondary peptic ulcer surgery group (4.5/1 female-to-male ratio). Although perioperative mortality was zero for both groups, patients undergoing secondary peptic ulcer surgery had a high number of complications (57% of patients had complications). Patients undergoing secondary peptic ulcer surgery scored lower in seven of the eight subclasses of the SF-36 questionnaire compared to their age-matched cohorts. In contrast, average Visick scores showed slight improvement for three out of four symptoms reported. Immediate postoperative complications were not related to long-term quality of life issues. Secondary peptic ulcer surgery is more prevalent in females than in males. Although secondary peptic ulcer surgery is partially effective in alleviating symptoms, quality of life is poor. (J GASTROINTEST SURG 2002; 6:396–402.)

KEY WORDS: Quality of life, recurrent, peptic ulcer, outcomes

Secondary peptic ulcer surgery is rare because of the success of a variety of medical therapies, in addition to the good outcome expected from primary peptic ulcer surgery. Despite its rarity, secondary peptic ulcer surgery is considered an alternative in treating symptoms associated with recalcitrant or recurrent peptic ulcer disease, or complications stemming from a primary peptic ulcer procedure. Previously it was suggested that secondary peptic ulcer surgery offered patients an improved quality of life as determined by Visick scores. However, this concept has been recently challenged given the morbidity associated with the procedure.[1] In the following report we review our experience with secondary peptic ulcer surgery and provide insight into to the impact of the procedure on the patient's quality of life as determined by the Medical Outcomes Study Questionnaire 36-Item Short Form Health Survey (SF-36).

PATIENTS AND METHODS

Between January 1, 1985, and December 31, 2000, thirty-five patients underwent secondary peptic ulcer surgery at Duke University Medical Center. Their medical records and clinic notes were analyzed for patient demographics, indications for surgery, primary peptic ulcer surgeries, secondary peptic ulcer surger-

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (poster presentation).

From the Department of Surgery (G.V.G.-S., J.M.R., H.F.S., J.P.G., M.F.K., S.B., T.N.P), Duke University Medical Center, Durham, North Carolina.

Reprint requests: Theodore N. Pappas, M.D., Duke University Medical Center, Box 3479, Durham, NC 27710. e-mail: pappa001@mc.duke.edu

1091-255X/02/$—see front matter
PII: S1091-255X(01)00029-4

ies, pathologic diagnosis, length of hospital stay, readmissions, and short-term outcomes. Long-term outcomes following surgical procedures were ascertained by way of a telephone interview. Long-term outcome variables included the impact of the surgery on the patient's gastrointestinal symptoms, the need for medication to relieve gastrointestinal symptoms, dietary habits, work status, overall satisfaction with the results of the surgical procedure, and quality of life as measured by the Medical Outcomes Study Questionnaire 36-Item Short Form Health Survey (SF-36).

To objectively quantify the severity of gastrointestinal complaints, a modified Visick grading system was used. Based on the severity of the symptoms and the need for medical therapy, Visick grades were as follows: grade 1 = no symptoms and no medical therapy; grade 2 = occasional symptoms but no medical therapy required; grade 3 = constant symptoms severe enough to require and respond to medical treatment; and grade 4 = severe and incapacitating symptoms unresponsive to medical treatment.

Quality of life was determined with the use of the SF-36. The SF-36 was chosen because it assesses health-related quality-of-life outcomes, namely, those known to be the most directly affected by disease and treatment. There are eight scales tested within the SF-36. Five scales (Physical Functioning, Role Physical, Bodily Pain, Social Functioning, and Role Emotional) define health status as the absence of limitation or disability, and three scales (General Health, Vitality, and Mental Health) measure a much wider range of negative and positive health states. The highest possible score for each scale is 100 and is achieved when patients perceive no limitations in their daily lives (based on the first five scales) and evaluate their health favorably (based on the latter three scales).[2]

SF-36 scores from patients undergoing secondary peptic ulcer surgery were compared to three age-matched control groups whose SF-36 scores were calculated after submitting them to the same SF-36 questionnaire. Group A was composed of 20 patients who had undergone a single peptic ulcer surgery for any complication of peptic ulcer disease. Patients in group A were age matched and had an average follow-up of 60 months. Group B included 20 patients who had undergone major curative resections for gastric adenocarcinoma, had no evidence of metastatic disease at presentation, and were potentially curable by surgical intervention. These patients were selected to represent age-matched cohorts with a total or near-total gastrectomy. The average follow-up for these patients was 60 months. Finally, group C was composed of a cohort of age-matched individuals without chronic disease. The SF-36 scores for group C have been previously published.[2] Data were analyzed using the Student's t test and P values of <0.05 were considered significant.

RESULTS

Patient Demographics

From January 1985 through December 2000, thirty-five patients underwent secondary peptic ulcer surgery at Duke University Medical Center. All patients requiring secondary peptic ulcer surgery did so despite treatment with H_2-blockers, proton pump inhibitors, and *Helicobacter pylori* clearance. A total of 20 patients were available for interviewing. Of the 35 patients undergoing secondary peptic ulcer surgery, 10 patients were lost to follow-up and five patients had died by the time the study was conducted. None of the deaths occurred in association with surgical procedures. The average follow-up was 60 months. The ages of the secondary peptic ulcer surgery patients ranged from 30 to 76 years (mean 49 years). Females comprised 82% of the 35 patients undergoing secondary peptic ulcer surgery outnumbering males by a 4.5 to 1 ratio.

The most common primary peptic ulcer surgery performed in the 35 secondary peptic ulcer surgery patients was a vagotomy and an antrectomy (53.1%). The second most common primary peptic ulcer surgery performed was a vagotomy with a pyloroplasty (18.8%). Other primary surgical procedures included partial gastrectomy (12.5%), vagotomy with a gastrojejunostomy (12.5%), and Graham patch (3.1%).

Secondary peptic ulcer surgeries were aimed at alleviating gastrointestinal symptoms that were refractory to medical therapy in 34 patients (97%). One patient (3%) developed a gastrocolic fistula following antrectomy and vagotomy, and was transferred to Duke University Medical Center for further treatment. Symptoms for which surgery was required included crippling abdominal pain (85%), severe heartburn (80%), unrelenting nausea and vomiting (80%), and severe diarrhea (50%).

All 35 secondary peptic ulcer surgery patients were extensively studied before surgery to determine the etiology of their gastrointestinal symptoms. Eleven percent of the patients underwent gastric emptying studies demonstrating a delayed emptying of solid foods. Nineteen patients (54%) underwent esophagogastroduoedenoscopy (EGD). During EGD 57% of patients had a visible ulcer either in the stomach or duodenum, and 25% had retained food within their stomachs. Ten patients (28%) had upper gastrointestinal series, which demonstrated the following: 3 of 10 patients with peptic ulcers; one patient with a gastrocolic fistula; one patient with gastric atony;

one patient with thickened mucosal folds; two patients with normal gastric remnant motility; one patient with a deformed pylorus; and one patient with a stenosed gastrojejunal anastomosis. A single CT scan of the abdomen and pelvis was performed in one patient with nausea and vomiting, which revealed a markedly dilated stomach with lack of contrast emptying consistent with gastric outlet obstruction.

Secondary peptic ulcer surgeries commonly involved some form of gastrectomy as a remedial surgery. Forty-five percent of those studied underwent a completion gastrectomy to alleviate their symptoms. Eleven percent of patients underwent a partial gastrectomy and 5.7% had a near-total gastrectomy. However, less extensive operations were also undertaken. Almost one fourth of the patients had a vagotomy and antrectomy, more than 10% had revisions and a vagotomy, and pyloroplasty was the operation of choice in one patient.

All specimens were carefully examined for pathologic findings that could explain the severe symptoms observed clinically. The most common histologic abnormalities included chronic gastritis (n = 16) and benign ulcers (n = 9). Other findings included a gastric antral mucosa with focal acute mucosal hemorrhage and mild acute serositis in two patients, a stomach with chronic inflammation, submucosal hemorrhage in one patient, a stomach with gastric mucosa containing areas of superficial hyperemia and hemorrhage, and a single gastric remnant with multiple areas of small gastric erosions; a single patient was found to have a stomach with prominent mucosal folds and fundic gland hyperplasia.

Although the 30-day hospital mortality rate was zero for the 35 patients undergoing secondary peptic ulcer surgery, nearly 60% of them had some type of complication. Twenty-three percent of them suffered an ileus. Nearly 20% developed an intra-abdominal abscess, and 80% of these leaks had an associated anastomotic leakage detected by postoperative radiologic contrast studies. Uncommon complications included superficial wound infections (8%), prolonged endotracheal intubation (8%), unplanned reintubation (5%), pneumothorax (5%), sepsis syndrome (5%), deep vein thrombosis (3%), pneumonia (3%), renal insufficiency (3%), and urinary tract infection (3%). As a result of these complications, the average hospital length of stay was 16.9 days (range 7 to 41 days).

Patients undergoing remedial surgeries were readmitted more frequently within a 3-month period after their discharge. The readmission rate for patients in the secondary peptic ulcer surgery group at 30, 60, and 90 days was 17.9%, 7.4%, and 3.7%, respectively.

Long-Term Outcomes

Gastrointestinal Symptoms. The impact that secondary peptic ulcer surgery had on gastrointestinal symptoms was determined by asking the patients to

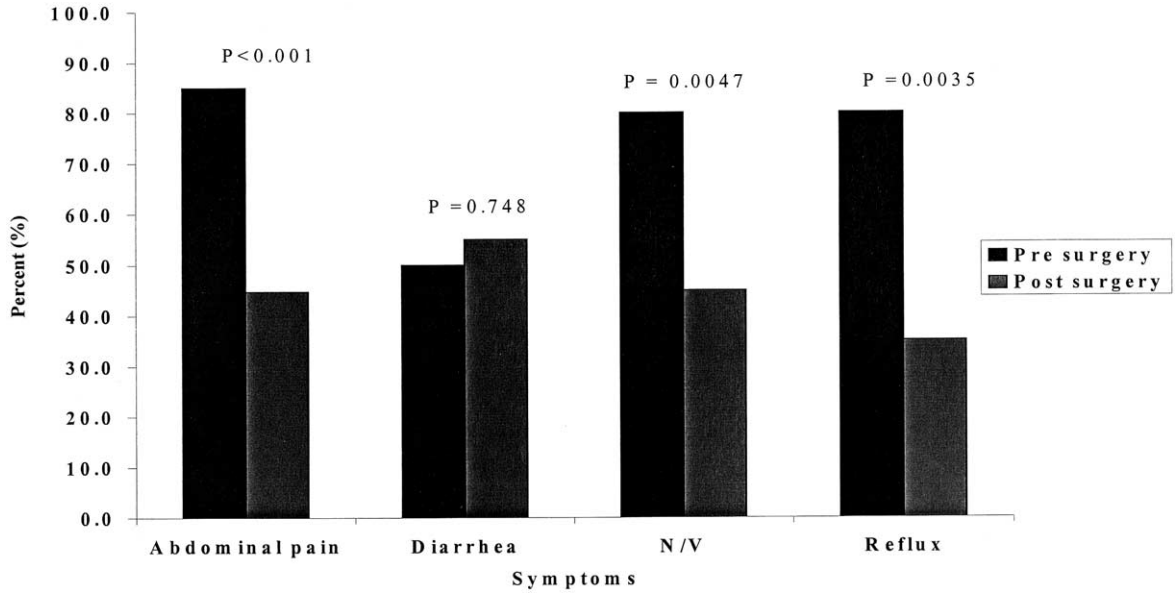


Fig. 1. Symptoms before and after surgery. Gastrointestinal symptoms elicited from patients during telephone interviews. Patients undergoing secondary peptic ulcer surgery (n = 20) noted an improvement in abdominal pain, nausea and vomiting, and reflux with surgery. Some patients did notice an increase in diarrhea, but this did not reach statistical significance. N/V = nausea and vomiting.

complete a gastrointestinal symptom questionnaire and answer the questions in relation to symptoms suffered before and after surgery. The most common symptom for which surgery was performed was abdominal pain (Fig. 1), which was reported by more than 80% of patients. With surgery this number was nearly halved ($P < 0.001$). Nausea and vomiting were also commonly reported and surgery had a similar impact on these symptoms as it did for abdominal pain. Diarrhea seemed to increase after surgical intervention, and this would not be surprising given that these patients would develop some type of dumping or malabsorption syndrome with the removal of the gastric reservoir (Fig. 1).

Visick Scores. With improvement in symptoms there was a detectable improvement in individual symptoms-associated Visick scores after surgery. A statistically significant improvement in Visick scores was detected for the symptoms of abdominal pain ($P < 0.001$), nausea and vomiting ($P = 0.005$), and reflux ($P = 0.004$) (Fig. 2). As described previously, the reported rate of diarrhea did increase with surgery; however, the impact that it had on the individual symptom-associated Visick score did not reach statistical significance ($P = 0.748$) (see Fig. 2).

Need for Medications to Alleviate Gastrointestinal Symptoms. When asked, the majority of secondary peptic ulcer surgery patients admitted using some type of medication to alleviate their gastrointestinal symptoms. With surgery the percentage of patients requiring medication was halved. Ironically, although 30% of patients who had diarrhea reported using medications before their surgery, none of them did so after surgery.

Nutritional Requirements. During the interview process, patients were asked about their dietary habits and what type of diet they were following during the perioperative period. The diets included solids, purees, or liquids, or the need for nutritional supplementation with total parenteral nutrition (TPN) or tube feeding. Before surgery, a large proportion of patients were only able to ingest a liquid diet because of their symptoms. Postoperatively there was a tendency for these patients to tolerate a solid diet; however, this did not reach statistical significance ($P = 0.94$). Although TPN and tube feeding were required as nutritional support in 45% and 10% of patients postoperatively, patients were gradually weaned from these feedings with the exception of one patient who was still dependent on TPN at follow-up.

Employment and Disability. At follow-up, 90% of patients admitted to holding a job preoperatively despite their disease. However, 10% of the group interviewed were on disability as a source of income. After surgery, 50% of those interviewed had returned to work in either a full-time or part-time position. The other half of the patients were disabled after surgery and required financial support to sustain themselves.

Overall Satisfaction. Despite the fact that a large proportion of patients became disabled after secondary peptic ulcer surgery, most of them were satisfied

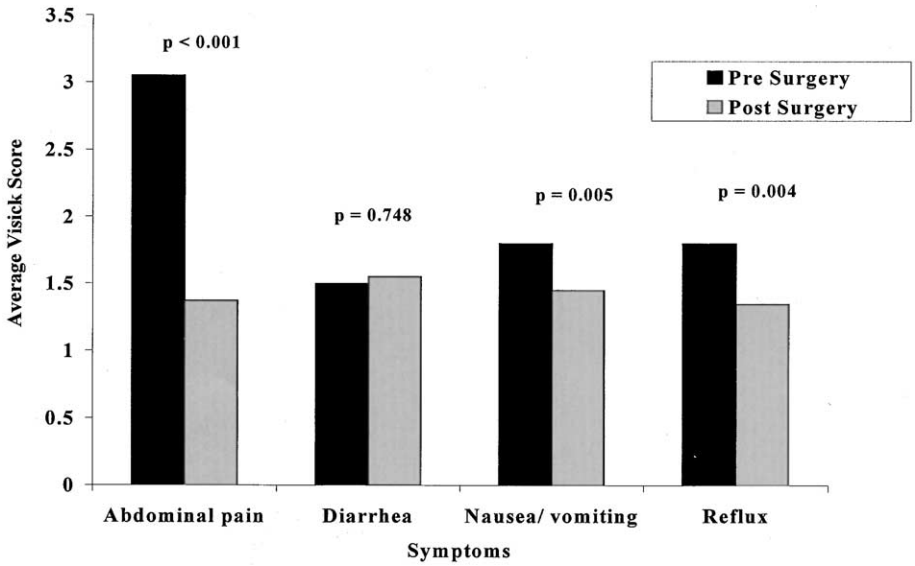


Fig. 2. Change in average Visick score with surgery. There was a significant improvement in most individual Visick scores with surgery in patients suffering of recalcitrant peptic ulcer disease (n = 20). However, although the reported diarrhea rate did increase, this did not seem to have a significant impact on the Visick score.

with their surgical results. Of the 20 patients interviewed, 16 (80%) admitted to being satisfied with their surgery.

Quality of Life. SF-36 scores in each subclass ranged from 0 to 100. A high score for a functional subclass indicates good functioning and few restrictions on quality of life, whereas the opposite is true for a low score. Average scores were used to compare intergroup differences. Overall, patients undergoing secondary peptic ulcer surgery scored worse than their age-matched cohorts (see Fig. 3). Worse scores were detected in those scales measuring physical health status: Physical Functioning, Role Functioning, and Bodily Pain. Patients undergoing secondary peptic ulcer surgery also scored poorly in those scales measuring both physical and mental well-being: Vitality and General Health. Regarding those scales that measure mental well-being, secondary peptic ulcer surgery patients scored worse than their cohorts in Social Functioning and Role Emotional. Surprisingly, although scores for Mental Health were lower than those in group A, average scores in the secondary peptic ulcer surgery group were better than those in group B. SF-36 scores on average were at least one standard deviation below the score for group C (Fig. 4).

DISCUSSION

Despite significant progress in gastric surgery over the past 50 years, recurrent ulceration after primary peptic ulcer surgery continues to be a challenging problem. Factors presumed to heighten the susceptibility to this disease include persistent use of aspirin products, smoking, and an inadequate first ulcer surgery.[3,4] Most reports suggested that recurrent and/or recalcitrant peptic ulcer disease is gender specific, affecting mainly males.[1,5–9] However, we and others have found that females are more prone to develop recurrent peptic ulcer disease requiring remedial peptic ulcer surgery.[10,11] Although there are diseases that certainly are gender specific, why females are more prone to develop recurrent or recalcitrant peptic ulcer disease is still unclear. It is possible that postsurgical gastric atony is more prevalent in females after primary peptic ulcer surgery, as has been previously suggested,[10–12] or that these patients suffered from a primary dysmotility disorder of the stomach and/or the small bowel to begin with rather than an acid-mucosa imbalance at their initial presentation.

Patients requiring a second peptic ulcer surgery are often middle aged (commonly in their 40s or 50s), have multiple gastrointestinal complaints despite maximal medical therapy, and require not only a second ulcer surgery but many times three or more procedures.[1,3,5–10]

Although mortality associated with reoperative interventions has diminished to nearly zero, secondary peptic ulcer surgery continues to be associated with significant morbidity.[11,13] As we have shown, more than half of all patients undergoing secondary peptic ulcer surgery develop some type of complication. Moreover 19% of patients undergoing secondary peptic ulcer surgery develop an intra-abdominal ab-

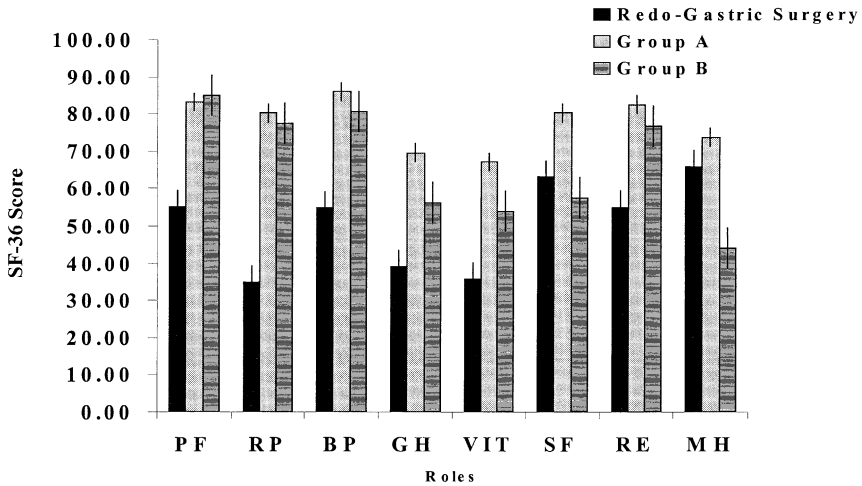


Fig. 3. Comparison of SF-36 scores between patients undergoing secondary peptic ulcer surgery (n = 20) and patients undergoing primary peptic ulcer surgery (n = 20) and patients undergoing gastrectomy for gastric carcinoma (n = 20). Patients undergoing secondary peptic ulcer surgery scored worst in seven of the eight subscales of the SF-36. PF = physical function; RP = role physical; BP = bodily pain; GH = general health; VIT = vitality; SF = social functioning; RE = role emotional; MH = mental health.

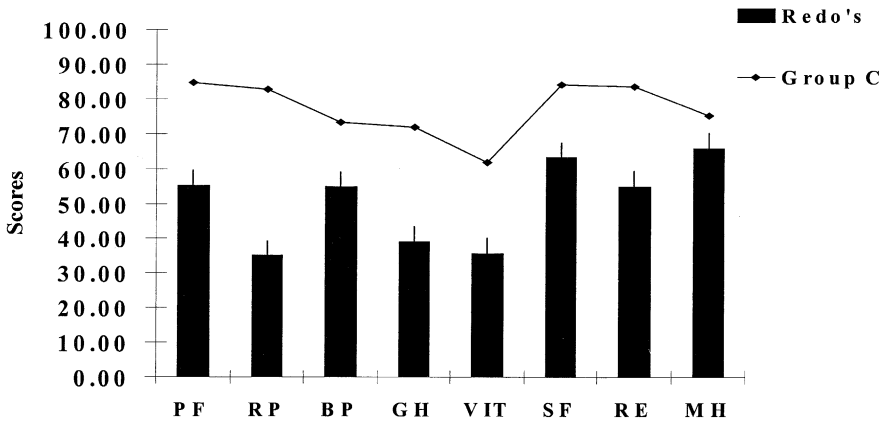


Fig. 4. Comparison of SF-36 scores between patients undergoing peptic ulcer surgery and an age-matched cohort of individuals without chronic disease. Secondary peptic ulcer surgery patients (n = 20) scored worse than aged-matched healthy individuals in all eight subscales of the SF-36. PF = physical function; RP = role physical; BP = bodily pain; GH = general health; VIT = vitality; SF = social functioning; RE = role emotional; MH = mental health.

scess. We believe that this rate reflects the 25% incidence of preoperative bezoar formation in our study group, which might have predisposed them to bacterial overgrowth in atonic stomachs, operations in contaminated fields, and a higher than expected rate of intra-abdominal abscesses.

With the added morbidity associated with secondary peptic ulcer surgeries comes a large economic burden. We have shown that patients after secondary peptic ulcer surgeries seem to remain hospitalized for long periods of time and have a high rate of readmission. Furthermore, in our experience secondary surgery ulcer patients took longer to return to work (data not shown) and a greater proportion were incapable of returning to work after surgery.

Despite these shortcomings, secondary peptic ulcer surgeries are effective procedures.[3,8] Most authors agree that when Visick grades are used as a measure of surgical success, there is an improvement in symptoms and most patients can be converted to a lesser Visick grade.[1,6,7,9,10] Our experience mirrors these reports, as Visick grades for most of our patients diminished after surgery.

However, the improvement in Visick grade seems not to equate with an improvement in quality of life, as might have been suggested in the past.[1] In our experience, with the use of a more specific measure of quality of life, the SF-36, most patients did not enjoy an improvement. In fact, their quality of life seemed to be worse than that of patients operated on for a primary peptic ulcer or gastric carcinoma. In addition, patients undergoing reoperation seemed to have a poorer quality of life than a healthy aged-matched population in the United States, even after several years had elapsed since their operations. The reason for this occurrence is theorized to be multifactorial. For instance, most patients with recalcitrant peptic ulcer disease suffer from chronic pain and are chronically dependent on narcotics to control the symptoms associated with their disease. This chronicity might lead to the development of psychological problems, which cannot be corrected by surgery. Although we did not ascertain what proportion of patients had concomitant psychiatric illnesses, others have suggested that this might explain the poor results in this patient population.[13] In fact, some investigators have even gone through the process of psychological testing before embarking on any surgical correction to weed out those individuals who do not suffer from recurrent or recalcitrant symptoms of peptic ulcer disease symptoms.[10–12]

CONCLUSION

Secondary peptic ulcer surgery seems to be more prevalent in females than in males. Although secondary peptic ulcer surgery is partially effective in alleviating symptoms associated with recurrent peptic ulcers complicating primary peptic ulcer surgery, quality of life is not returned to that of a normal healthy individual.

REFERENCES

1. Heppell J, Bess MA, McIlrath DC, Dozois RR. Surgical treatment of recurrent peptic ulcer disease. Ann Surg 1983; 198:1–4.
2. Ware JE. SF-36 Health Survey: Manual and Interpretation Guide. Boston: The Medical Outcomes Trust, 1993.

3. Browder W, Thompson J, Youngberg G, Walters D. Delayed ulcer recurrence after gastric resection: A new postgastrectomy syndrome? Am Surg 1997;63:1091–1096.
4. Hirchowitz BI, Lanas A. Intractable upper gastrointestinal ulceration due to aspirin in patients who have undergone surgery for peptic ulcer disease. Gastroenterology 1998;114: 883–892.
5. Muscroft TJ, Taylor EW, Deane SA, Alexander-Williams J. Reoperation for recurrent peptic ulceration. Br J Surg 1981; 68:75–76.
6. Pietri P, Gabrielli F, Pellis G. Reoperation for recurrent peptic ulcer. Int Surg 1983;68:301–305.
7. Hoffman J, Shokouh-Amiri MH, Klarskov P, et al. Gastrectomy for recurrent ulcer after vagotomy: Five- to nineteen-year follow-up. Surgery 1986;99:517–522.
8. Jaffe BM, Newton WT, Judd DR, Ballinger WF. Surgical management of recurrent peptic ulcers. Am J Surg 1969; 117:214–221.
9. Gustavsson S, Kelly KA. Total gastrectomy for benign disease. Surg Clin North Am 1987;67:539–551.
10. Eckhauser FE, Knol JA, Raper SA, Guice KS. Completion gastrectomy for postsurgical gastroparesis syndrome. Ann Surg 1988;208:345–353.
11. Forstner-Barthell AW, Murr MM, Nitecki S, et al. Near-total completion gastrectomy for severe postvagotomy gastric stasis: Analysis of early and long-term results in 62 patients. J GASTROINTEST SURG 1999;3:15–21.
12. Sawyer JL. In discussion: Completion gastrectomy for postsurgical gastroparesis syndrome. Ann Surg 1988;208:352–353.
13. Farahmand M, Sheppard BC, Deveney CW, et al. Long-term outcome of completion gastrectomy for nonmalignant disease. J GASTROINTEST SURG 1997;1:182–187.

Enterocyte Response to Ischemia Is Dependent on Differentiation State

Brian F. Hinnebusch, M.D., Qing Ma, M.D., J. Welles Henderson, B.A., Aleem Siddique, M.D., Sonia Y. Archer, M.D., Richard A. Hodin, M.D.

Enterocytes at the tips of microvilli are more sensitive to an ischemic insult than those cells residing in the crypts, an effect thought to be due to a relative lack of collateral flow. We speculated that this increased cellular sensitivity to ischemia might be an intrinsic feature of the cells related to their differentiated phenotype. To test this hypothesis, enterocyte response to ischemia was determined using both in vivo and in vitro models. For the in vivo studies, male Sprague-Dawley rats underwent laparotomy, and small intestinal ischemia was induced by clamping the superior mesenteric artery for 30 or 60 minutes, after which reperfusion was allowed for various time points up to 4 days. Injury was assessed histologically, as well as with Northern blots, probing for the enterocyte differentiation markers intestinal alkaline phosphatase and lactase, as well as the gut-epithelial marker villin. Mucosal changes consistent with ischemia/reperfusion injury were evident—that is, a rapid inflammatory response followed by progressive villus cell loss beginning at the tips and progressing to the crypts, depending on the degree of insult, with an eventual return to normal microanatomy. Intestinal alkaline phosphatase and lactase were lost immediately after ischemia and returned with reperfusion, confirming that the differentiated cells are particularly sensitive to ischemic injury. The in vitro studies employed two separate models of enterocyte differentiation: sodium butyrate–treated HT-29 cells and Caco-2 cells maintained for 7 days after confluence. In both models, undifferentiated and differentiated cells were subjected to treatment with 2-deoxyglucose and oligomycin-A (in vitro model of ischemia) and apoptosis was assessed by fluorescence-activated cell sorting analysis. Differentiation of both cell lines resulted in a significantly greater apoptotic response to ischemia compared to undifferentiated cells exposed to an identical insult. We conclude that differentiated enterocytes may be inherently more sensitive to ischemia-induced injury than their undifferentiated counterparts. These findings call into question the popularly held belief that villus tip cells are more susceptible to ischemia because of their location relative to the microvascular anatomy. (J GASTROINTEST SURG 2002;6:403–409.)

KEY WORDS: Small intestine, ischemia, apoptosis, differentiation

The small intestinal lumen is lined by a simple columnar epithelium, which is continually regenerated and has a turnover time of 3 to 6 days.[1] This constant renewal of the gut mucosa is critical for the maintenance of its structural and functional integrity. The rapid proliferation and replacement of sloughed cells is accomplished by the coordinated and highly regulated processes of replication, migration, differentiation, and apoptosis.[2,3] A population of rapidly dividing pluripotent stem cells located in the crypts of Leiberkuhn give rise to four distinct cell lineages, of which the enterocyte is the most common, comprising approximately 95% of the overall small bowel epithelial population.[4] As these stem cells divide, they migrate upward along the crypt-villus axis, withdraw from the cell cycle, differentiate, and eventually undergo apoptosis and are sloughed into the lumen.[5] The mechanisms governing this highly ordered progression from rapidly dividing pluripotent stem cell to terminally differentiated, apoptotic cell are not well understood but are clearly innate characteristics of the intestinal epithelium, which can be modified by a variety of physiologic and pathophysiologic stimuli.[6–8]

Gut ischemia is commonly observed in several clinical settings, among which are acute occlusive or

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (oral presentation).

From the Beth Israel Deaconess Medical Center/Massachusetts General Hospital (B.F.H., Q.M., J.W.H., A.S., S.Y.A., R.A.H.), Harvard Medical School, Boston, Massachusetts.

Supported by grants DK47186, DK02527, and DK50623 from the National Institutes of Health.

Reprint requests: Richard A. Hodin, M.D., Massachusetts General Hospital, Department of Surgery, 504 Gray Building, 55 Fruit St., Boston, MA 02114. e-mail: rhodin@partners.org

1091-255X/02/$—see front matter
PII: S1091-255X(01)00076-2

low-flow states, transient intestinal ischemia associated with vascular bypass procedures requiring cross-clamping of the aorta, and trauma patients being resuscitated from hemorrhagic shock. It is known that ischemia-induced injury to the gut begins in the mucosa and progresses outwardly to the serosa.[9,10]

The cells at the tips of the villi are particularly sensitive to an ischemic insult, an observation thought to be the result of their location at the end distribution of a central arteriole, which leads to a lower oxygen tension compared to the crypt.[11] The gut epithelium has been shown to respond to ischemia by undergoing apoptosis rather than necrosis.[12–14] Because, under normal conditions, differentiated enterocytes (or villus cells) are programmed to undergo apoptosis, and these same cells are those which are most sensitive to an ischemic insult, we speculated that their increased sensitivity to ischemia may be an intrinsic property of the cells related to their differentiation state. The specific aim of this study was to demonstrate that differentiated enterocytes are more susceptible to ischemia-induced apoptosis irrespective of blood flow.

MATERIAL AND METHODS

In Vivo Studies

Male Sprague-Dawley rats (200 to 250 g) were obtained from Charles River (Wilmington, Massachusetts) and maintained on a diet of standard chow diet with water ad libitum, in accordance with the institutional guidelines set forth by the animal welfare committee of the Beth Israel Deaconess Medical Center. The animals were fasted but given water ad libitum for 1 day before the experiment. Anesthesia was induced with an intraperitoneal injection of pentobarbital (40 mg/kg). After ensuring that an adequate level of anesthesia was being maintained, laparotomy was performed and the superior mesenteric artery (SMA) was occluded with a microvascular clamp (George Tiemann and Co., Happauge, New York) for a period of 30 or 60 minutes, after which the clamp was removed (time 0) and the incision was closed with simple interupted 3-0 silk sutures. The animals were then allowed to recover and subsequently maintained on a diet of standard chow with water ad libitum. At 6 and 24 hours after ischemia, the animals were killed by an overdose of pentobarbital (80 mg/kg). Segments of jejunum (10 cm beginning at the ligament of Treitz and running distally) and ileum (10 cm beginning at the ileocecal valve and running proximally) were harvested and fixed in 37% formaldehyde for histologic examination or processed for Northern blot analyses.

Histologic Findings

Segments of rat small intestine were imbedded in paraffin and then serially sectioned, fixed, and stained with hematoxylin and eosin following standard histologic protocols. The crypt-villus height was determined by an independent observer in both the control and postischemia groups (n = 10) from randomly selected sections (double-blind design) at ×100 magnification using a calibrated ocular-grid eyepiece (Nikon, Tokyo, Japan).

Northern Blot Analysis

Total RNA was extracted from rat small intestine using the guanidium thiocyanate method.[15] Northern blot analyses were performed by loading 20 μg of RNA per lane on an agarose-formaldehyde gel, separating by electrophoresis, transferring onto nitrocellulose membranes, and baking for 2 hours at 80° C. Equal loading of RNA per lane was confirmed by examination of ethidium bromide–stained gels. Complementary DNA probes were ^{32}P radiolabled to a specific activity of approximately 5×10^8 cpm/μg DNA according to the technique of Feinberg and Vogelstein.[16] The intestinal alkaline phosphatase (IAP) probe is a 1.9 kb Pst1 fragment derived from the human IAP cDNA[17] and the villin probe is a 530 bp fragment from the human cDNA and was provided by Dr. M. Arpin.[18] The lactase probe is a 1.8 kb EcoRI/PstI fragment derived from the rat cDNA and was provided by Dr. Richard Grand.[19] The actin probe is a 1.0 kb PstI fragment derived from the mouse β-actin cDNA.[20] Hybridizations were carried out in 5× saline sodium citrate (1× SSC = 3 mol/L NaCl, 0.3 mol/L SCC)/50% formamide/1% sodium dodecyl sulfate (SDS) (Sigma, St. Louis, Missouri) at 42° C. The washing conditions were 2× SSC/0.1% SDS at 50° C.

Cell Culture

HT-29 and Caco-2 cells were obtained from the American Type Culture Collection (Manassas, Virginia) and maintained in standard Dulbeccos's modified eagle medium (Gibco BRL, Rockville, Maryland) with 10% fetal bovine serum, 2 mmol/L glutamine, and 100 U/ml penicillin-streptomycin (Bio-Whittaker, Walkersville, Maryland) at 37° C and 5% CO_2. Cells were passaged and experiments performed at 70% confluence except in the case of the postconfluent studies as indicated. Medium was changed every 3 days and just before each experiment. Differentiation was induced in the HT-29 cells by adding sodium butyrate (Sigma) to the medium to a final concentration of 5 mmol/L

for 24 hours and in the Caco-2 cells by maintaining them for 7 days after they had reached confluence.

In Vitro Ischemia

Cells were washed once with high–phosphate buffered Ringer's solution containing glucose (HPBR+ 5 mmol/L HEPES, 5 mmol/L KCl, 3.33 mmol/L NaH_2PO_4, 0.83 mmol/L Na_2HPO_4, 1 mmol/L $MgCl_2$, 135 mmol/L NaCl, 1 mmol/L $CaCl_2$, 10 mmol/L glucose, Sigma) and then incubated in HPBR+ for 30 minutes at 37° C and 5% CO_2, after which they were washed once with high–phosphate buffered Ringer's solution without glucose (HPBR−) and divided into two treatment groups: nonischemic control cells and ischemic cells. The ischemic cells were incubated with 10 mmol/L 2-deoxyglucose and 1 μmol/L oligomycin-A (Sigma) in HPBR− (OLI/DOG cocktail) for various time points, while the control cells were incubated in HPBR+ for the same time periods, with all incubations at 37° C and 5% CO_2. This ischemia protocol has previously been shown to result in marked but reversible adenosine triphosphate depletion.[21] After treatment, both groups of cells were washed three times in phosphate-buffered saline solution (PBS; pH 7.4, Gibco) and returned to standard medium until harvest for fluorescence-activated cell sorter (FACS) analysis.

Apoptosis Analysis

Cells were seeded at a density of 1×10^6 per well in six-well cluster plates (Becton-Dickinson, Franklin Lakes, New Jersey), and chemically-induced ischemia was administered as indicated. The cells were then returned to standard medium for 24 hours, after which they were collected in trypsin (Bio-Whittaker), washed with PBS (pH 7.4), and fixed in 70% ethanol (Aaper, Shelbyville, Kentucky) overnight. The following day, the cells were centrifuged at 1000 rpm for 10 minutes, resuspended in 50 μg/ml propidium iodide (Sigma) in PBS, and immediately subjected to flow cytometry optimized for propidium iodide using an FACS scan (Becton-Dickinson). Appropriate settings of forward and side scatter gates were used to examine 10,000 cells per sample. Results were analyzed with Cell Quest (Becton-Dickinson) and Modfit (Verity Software House, Topsham, Maine) software.

Statistical Analysis

Statistical analysis was carried out by means of Student's *t* test and two-way analysis of variance where appropriate, with $P < 0.05$ considered statistically significant.

RESULTS

Villus tip cells are more sensitive to an ischemic insult. The ischemia induced by SMA occlusion resulted in mucosal sloughing accompanied by an intense inflammatory infiltrate during the reperfusion period. The severity of injury was proportional to the degree of ischemic time (30 or 60 minutes) and was demonstrated by the progressive loss of the villi beginning at the tips and moving toward the crypt base. Segments of jejunum and ileum were studied and compared to nonischemic control specimens from the corresponding segment in each group. Fig. 1, *A* shows the histologic damage in a representative section of jejunum harvested after 6 hours of reperfusion following an ischemic insult of 60 minutes. By 24 hours the microanatomy returns to normal. A quantitative analysis of the damage is depicted in Fig. 1, *B*. In both the jejunum and ileum, there is a significant decrease in the crypt-villus heights at 6 hours after an ischemic time of 30 minutes (16.8% and 25%, respectively; $P < 0.005$, $n = 10$) compared to the respective nonischemic control specimens. Sixty minutes of ischemic time resulted in an even greater decrease (51.1 and 98.1%, respectively; $P < 0.005$). After 24 hours of reperfusion, the crypt-villus heights return to preischemia control levels.

Differentiated enterocytes are more sensitive to an ischemic insult in vivo. To confirm that it is the loss of the differentiated enterocytes that results in the decrease in the crypt-villus height, we subjected the postischemia small intestines to Northern blot analyses. Fig. 2 shows blots of RNA taken from rat small intestines harvested at 6 and 24 hours of reperfusion after an ischemic insult of 60 minutes. The control rats underwent a sham laparotomy, which was closed after 60 minutes. The differentiation markers IAP and lactase are shown to be expressed in the control intestines (lane C). These markers, which are only expressed by differentiated enterocytes, are lost at 6 hours of reperfusion as a result of the ischemia, indicating the loss of differentiated enterocytes from the mucosa. At 24 hours these markers return to near-normal levels of expression, paralleling the time course of the histologic damage. Villin expression, which is greater in differentiated enterocytes but also occurs in the undifferentiated cells, is significantly attenuated at 6 hours but not completely lost, indicating that viable enterocytes remain within the crypts. As is the case for IAP and lactase, villin expression also returns to normal by 24 hours. Actin is included as a control for equal loading of RNA in each lane.

Differentiated cells are more sensitive to ischemia-induced apoptosis in vitro. It has been shown

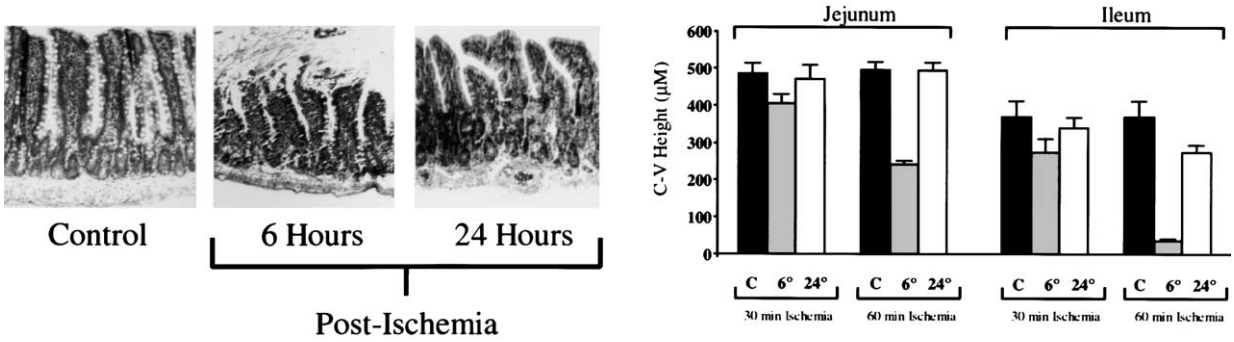


Fig. 1. Rat small intestinal histologic response to an ischemic insult of 60 minutes. **A,** At 6 hours of reperfusion, there is an intense inflammatory infiltrate and marked sloughing of the villus tips followed by a return to normal microanatomy at 24 hours. **B,** The loss of the villus tips is demonstrated by the decrease in the crypt-villus height, which also returns to preischemia control values at 24 hours. Shown are the average measurements (n = 10) taken from representative sections of jejunum and ileum at 6 and 24 hours after ischemic times of 30 and 60 minutes.

in vivo that enterocytes respond to transient ischemia by undergoing apoptosis, an active process that requires new protein synthesis.[12–14] A minimal degree of ischemia-induced apoptosis was seen in the undifferentiated (preconfluent) Caco-2 cells (1.1-fold vs. control, $P < 0.005$; Fig. 3). In contrast, differentiated cells (postconfluent) undergo a significantly greater apoptotic response to ischemia (7-fold vs. control, $P < 0.005$; Fig. 3). Because the in vitro model removes the cells from a microcirculation, this result suggests that the increased cellular sensitivity to ischemia may be an intrinsic feature of differentiated enterocytes.

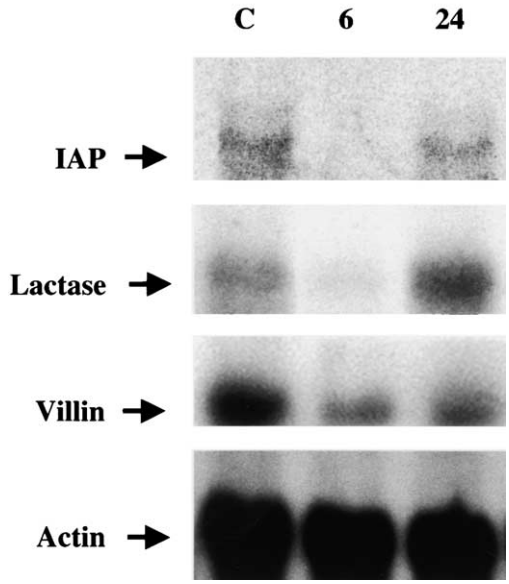


Fig. 2. Northern blot analysis of rat jejunal mucosal RNA probing for the enterocyte differentiation markers intestinal alkaline phosphatase (*IAP*) and lactase, as well as the gut epithelial marker villin. Both IAP and lactase are lost at 6 hours after an ischemic insult of 60 minutes. Villin, which is more highly expressed in the differentiated enterocytes, is significantly decreased but not lost. The markers return to preischemia control values by 24 hours. Actin is included as a control for equal RNA loading. The blot shown is representative of three separate experiments.

Finally, we examined HT-29 cells using the differentiation agent sodium butyrate. The HT-29 cell line was employed to independently verify the findings observed in the Caco-2 cell line. Butyrate itself caused an increase in apoptosis (Fig. 4), which complicates the interpretation of the ischemia-induced apoptosis. However, results with this second, independent in vitro model system were similar to those seen with the Caco-2 cell model. Indeed, differentiated HT-29 cells were more sensitive to ischemia-induced apoptosis than their undifferentiated counterparts. Fig. 4 demonstrates that there is a 14.6-fold ($P < 0.005$) increase in apoptosis in ischemic, differentiated cells compared to nonischemic, undifferentiated cells. In contrast, ischemic, undifferentiated cells demonstrated only a 2.6-fold increase in apoptosis ($P < 0.005$) when compared to nonischemic, undifferentiated cells.

We next compared ischemia-induced apoptosis in pre- and postconfluent HT-29 cells. Because postconfluence does not cause differentiation in these cells, this experiment was designed to test whether the sensitivity to ischemia in Caco-2 cells was truly related to the differentiation process, as opposed to being a function of altered growth. Pre- and postconfluent HT-29 cells were equally sensitive to ischemia-induced apoptosis (Fig. 3), supporting the conclusion that it is the differentiating effects of

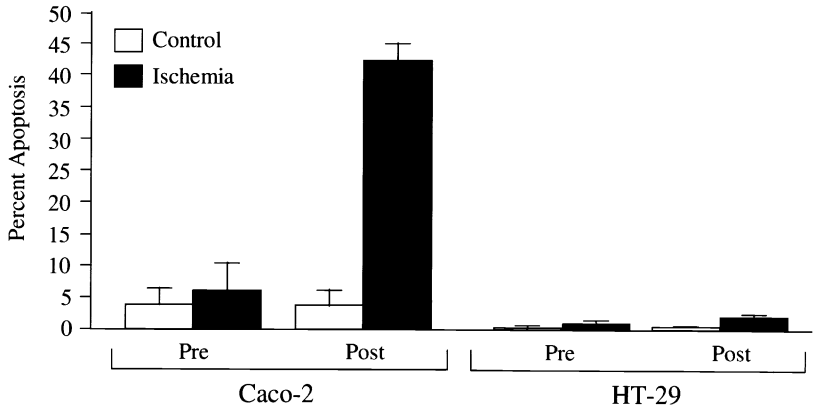


Fig. 3. Effect of differentiation on ischemia-induced apoptosis in Caco-2 cells. Undifferentiated (preconfluent) cells exhibited a small apoptotic response to a transient chemically induced ischemic insult of 3 hours. The baseline rate of apoptosis (control) in the differentiated (postconfluent) cells is unchanged compared to the undifferentiated cells; however, differentiation results in a dramatic increase in ischemia-induced apoptosis (7-fold vs. 1.1-fold, $P < 0.005$, n = 3). Postconfluence, which does not cause differentiation in HT-29 cells, did not increase the baseline rate of apoptosis or result in a higher rate of ischemia-induced apoptosis in this cell line.

post-confluence in Caco-2 cells that result in the increased sensitivity to ischemia-induced apoptosis.

DISCUSSION

The processes of gut mucosal epithelial cell growth arrest, migration, differentiation, and apoptosis are critical for the maintenance of small intestinal structure and function. Perturbations of any of these processes can lead to the clinical sequelae of diarrhea, malabsorption, and impaired barrier function, as is commonly observed in the critical care setting. An understanding of the highly coordinated mechanisms that regulate gut mucosal turnover under normal conditions may provide insight into how these processes are modified in response to pathophysiologic stimuli, and in turn may lead to specific interventions that could be used to preserve small intestinal structure and function in the face of a variety of insults.

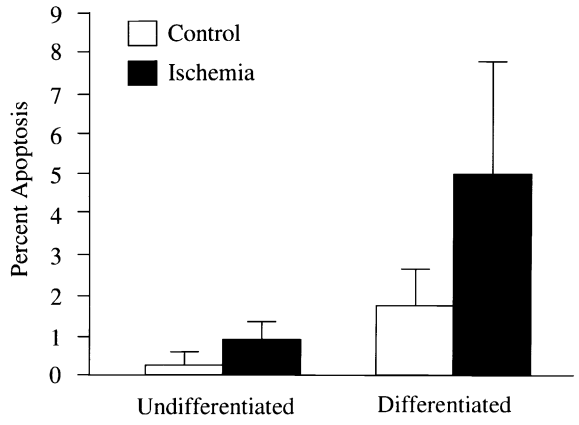


Fig. 4. Effect of differentiation on ischemia-induced apoptosis in HT-29 cells. As was the case with the Caco-2 cell line, undifferentiated HT-29 cells demonstrated a relatively small apoptotic response to the transient ischemia. Differentiation with sodium butyrate caused an increased rate of apoptosis and resulted in a significantly large amount of ischemia-induced apoptosis (14.6-fold vs. 2.6-fold increase, $P < 0.005$, n = 9).

Transient intestinal ischemia is commonly encountered in clinical practice, particularly in surgical critical care. The present studies confirm the mucosal damage that occurs in the gut in response to ischemia. That is, with increasing ischemic time, the small intestinal villi begin to slough, beginning at the tips and moving toward the crypts. Cell loss results not from necrosis, as might be expected, but rather from apoptosis,[12–14] an active process that requires new protein synthesis. The observation that enterocytes residing at the tips of the villi are the first to undergo apoptosis in response to transient ischemia, coupled with the knowledge that these same cells are, under normal conditions, destined for programmed cell death as part of their normal life span, suggested that these differentiated enterocytes might be "primed" for an apoptotic response to stressful stimuli such as ischemia.

We have shown that it is indeed the differentiated enterocytes that are preferentially lost because of an ischemic insult in vivo. The differentiation markers IAP and lactase are lost after ischemia, whereas the expression of villin, which is expressed by both dif-

ferentiated and undifferentiated enterocytes, is just attenuated after the same amount of ischemia/reperfusion. Taken together, these findings indicate that it is the differentiated enterocytes that are lost while the undifferentiated cells remain to restore the villi to their normal microanatomy when perfusion is restored.

It has generally been assumed that the increased sensitivity to ischemia exhibited by the differentiated enterocytes is related to the fact that these cells reside at or near the villus tip at the end of the vasculature without collateral flow. By employing two in vitro model systems, however, we were able to determine the response of cells to equivalent degrees of ischemia in the absence of a microcirculation. This approach removes the confounding effects of relative variations in blood flow based on microanatomic location. Using the two independent models of enterocyte differentiation, we have shown that differentiated cells undergo a significantly greater apoptotic response to ischemia than undifferentiated cells. These results suggest that the enterocyte's response to an ischemic insult may depend, at least in part, on intrinsic cellular factors based on differentiation state.

The two in vitro models, pre-/postconfluent Caco-2 and butyrate-treated HT-29 cells, have both been extensively characterized by our laboratory and others.[22, 23] In both models a variety of differentiation characteristics are seen, including growth arrest, induction of cell cycle inhibitors followed by markers of differentiation and morphologic features such as microvilli. We now add increased sensitivity to ischemia to the list. Indeed, in both cell lines a dramatic increase in ischemia-induced apoptosis was observed in the differentiated cells compared to their undifferentiated counterparts. This was readily apparent in the Caco-2 cells, as the undifferentiated (preconfluent) cells had only a minimal increase in apoptosis in response to ischemia, whereas the differentiated cells (postconfluent) exhibited a marked increase in apoptosis in response to the same ischemic insult. In the case of the HT-29 cells, the differentiating agent butyrate caused an increase in apoptosis, which complicates interpretation of ischemia-induced apoptosis in the differentiated cells. In the assay used to generate these data, apoptosis is a binary function. That is, either a cell is apoptotic or it is not. Thus cells that undergo apoptosis as a result of butyrate treatment are already counted as apoptotic; they can not become "more" apoptotic. Therefore the difference between the ischemic and control groups in the butyrate-treated cells represents a subpopulation of cells undergoing "new" apoptosis, since the cells in this subpopulation did not become apoptotic as a result of butyrate treatment or ischemia alone but rather only when both factors were present. For control purposes, we employed pre- and post-confluent HT-29 cells, because these cells do undergo growth arrest but do not differentiate. The fact that pre- and postconfluent HT-29 cells exhibited similar ischemia-induced apoptotic responses strongly supports our hypothesis that it is the differentiation process that specifically makes the cells more sensitive to ischemia.

The present results indicate that the undifferentiated enterocytes are relatively more resistant to an ischemic insult, suggesting that a novel therapeutic approach to protect the small bowel from transient ischemia-induced damage might be possible (i.e., maintaining gut epithelial cells in an undifferentiated state before or during ischemia). Alternatively, future investigation may reveal the links between differentiation-induced and ischemia-induced apoptosis, providing potential targets to manipulate in order to protect the small bowel mucosa from ischemic damage. Such potential interventions would undoubtedly be valuable in settings such as vascular procedures or intestinal transplantation where gut ischemia is planned in advance.

CONCLUSION

Differentiated enterocytes are more sensitive to an ischemic insult due, at least in part, to their more differentiated phenotype. As such, this increased susceptibility to ischemia-induced apoptosis appears to be an intrinsic feature of the cells based on differentiation state rather than an entirely nonspecific response based on relative oxygen tension. These results suggest that further investigation into the link(s) between enterocyte differentiation, apoptosis, and ischemia-induced apoptosis may identify targets to manipulate in order to protect the small bowel from an ischemic injury.

We wish to thank the laboratory of Dr. Jeffery B. Matthews for help in establishing the in vitro model of chemically induced ischemia.

REFERENCES

1. Potten CS. Epithelial cell growth and differentiation. II. Intestinal apoptosis. Am J Physiol 1997;273:253–257.
2. Gordon JI, Schmidt GH, Roth KA. Studies of intestinal stem cells using normal, chimeric and transgenic mice. FASEB J 1992;6:3039–3050.
3. Traber PG. Diferentiation of intestinal epithelial cells: Lessons from the study of intestine-specific gene expression. J Lab Clin Med 1994;123:467–477.

4. Cheng H, Leblond CP. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian theory of the origin of the four epithelial cell types. Am J Anat 1974;141:537–562.
5. Gavriele Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in-situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992;119:493–501.
6. Gordon JI. Intestinal epithelial differentiation: New insights from chimeric and transgenic mice. J Cell Biol 1989; 108:1187–1194.
7. Williamson RC. Intestinal adaptation (first of two parts). Structural, functional and cytokinetic changes. N Engl J Med 1978;298:1393–1402.
8. Williamson RC. Intestinal adaptation (second of two parts). Mechanisms of control. N Engl J Med 1978;298:1444–1450.
9. Mitsudo S, Brandt LJ. Pathology of intestinal ischemia. Surg Clin North Am 1992;72:43–63.
10. Robinson JW, Mirkovitch V, Winistorfer B, Saegesser F. Response of the intestinal mucosa to ischemia. Gut 1981; 22:512–27.
11. Kampp M, Nilsson NJ. Extravascular shunting of oxygen in the small intestine of the cat. Acta Physiol Scand 1968;72: 396–403.
12. Noda T, Iwarkiri R, Fujimoto K, Matsuo S, Aw JY. Programmed cell death induced by ischemia-reperfusion in rat intestinal small mucosa. Am J Physiol Gastrointest Liver Physiol 1998;274:270–276.
13. Ikeda H, Suzuki Y, Suzuki M, Koike M, Tamura J, Tong J, Nomura M, Itoh G. Apoptosis is a major mode of cell death caused by ischaemia and ischaemia/reperfusion injury to the rat intestinal epithelium. Gut 1998;42:530–537.
14. Sha KA, Green CJ. Characterization of apoptosis in intestinal ischaemia-reperfusion injury—a light and electron microscopic study. Int J Exp Path 1997;78:355–363.
15. Chirgwin JM, Przybla AE, MacDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 1979;18:5294–5299.
16. Feinberg AP, Vogelstein B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Bioch 1983;132:6–13.
17. Henthorn PS, Raducha M, Edwards YH, Weiss MJ, Slaughter C, Lafferty MA, Harris H. Nucleotide and amino acid sequences of human intestinal alkaline phosphatase: Close homology to placental alkaline phosphatase. Proc Natl Acad Sci USA 1987;84:1234–1238.
18. Pringault E, Arpin M, Garcia A, Finidori J, Louvard D. A human villin cDNA clone to investigate the differentiation of intestinal and kidney cells in vivo and in culture. EMBO J 1986;5:3119–3124.
19. Buller HA, Kothe MJC, Goldman DA, Grubman SA, Sasak WV, Matsudaira PT, Montgomery RK, Grand RJ. Coordinate expression of lactate-phlorizin hydrolase mRNA and enzyme levels in rat intestine during development. J Biol Chem 1990;265:6978–6983.
20. Cleveland DW, Lopata MA, McDonald RJ, Conan NJ, Rutter WJ, Kirschner MW. Number and evolutionary conservation of α- and β-tubulin and cytoplasmic β- and γ-actin genes using specific cloned cDNA probes. Cell 1980;20: 95–105.
21. Matthews JB, Smith JA, Tally KJ, Menconi MJ, Nguyen H, Fink MP. Chemical hypoxia increases junctional permeability and activates electrogenic ion transport in human epithelial monolayers. Surgery 1994;116:150–157.
22. Hodin RA, Meng S, Archer S, Tang R. Cellular growth state differentially regulates enterocyte gene expression in butyrate-treated HT-29 cells. Cell Growth Diff 1996;7:647–653.
23. Pinto M, Robine-Leon S, Appay M, Kedinger M, Triadou N, Dussaulx E, Laeroix B, Simon-Assmann P, Maffen K, Fogh J, Zweibaum A. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol Cell 1983;47:323–330.

Invited Discussion—Expert Commentator

Keith A. Kelly, M.D. (Scottsdale, AZ): Dr. Hinnebusch's presentation zeroed in on an important problem—the ischemic gut. All of us have struggled with this through the years. We have tried to identify the ischemic gut in the early stages of ischemia and to prevent damage from the ischemia. Dr. Hinnebusch points out quite clearly that the crypt enterocytes intrinsically are not as susceptible to ischemia as those on the villous tips. Perhaps this is a good thing. The crypt enterocytes, being protected or being more resistant, can then regenerate the cells that eventually migrate out to the tips. Dr. Hinnebusch did show a beautiful slide showing that about 20 hours after ischemia, as I recall, the villous tip cells were pretty much back to normal.

How does one protect the cells on the tips of the villi to prevent damage from ischemia? Because the cells are exposed to the lumen, one thought that crossed my mind was to perfuse the lumen of the gut with oxygen itself or, perhaps fluorocarbons to which oxygen was loosely coupled to make oxygen available to the cells. However, identifying states in which ischemia is likely to occur, preventing the ischemia, and restoring the circulation would likely be the directions to go.

Epidermal Growth Factor Regulation of System L Alanine Transport in Undifferentiated and Differentiated Intestinal Caco-2 Cells

Ming Pan, M.D., Ph.D., Wiley W. Souba, Jr., M.D., Sc.D., Anne M. Karinch, Ph.D., Cheng-Mao Lin, Ph.D., Bruce R. Stevens, Ph.D.

Epidermal growth factor (EGF) in intestinal lumen regulates many gut epithelial cell functions. Influenced by growth factors at various differentiation stages, enterocytes execute the major task of absorbing nutrient amino acids. The purpose of this study was to investigate the effects of EGF on Na^+-independent L-alanine transport in intestinal epithelial cells. Na^+-independent [^{3}H]-L-alanine transport was measured in the differentiating Caco-2 cells. In both the undifferentiated and differentiated states, L-alanine uptake occurred via a single saturable Na^+-independent system L plus simple passive diffusion. System L activity decreased as the cells progressed from the undifferentiated to the differentiated state. Prolonged incubation with EGF (>30 hours) resulted in a 70% increase in system L activity in both undifferentiated and differentiated cells. EGF stimulated the system L V_{max} without affecting K_m. System L activity stimulation was inhibited by chelerythrine chloride, cycloheximide, or actinomycin D. These data suggest that intestinal epithelial cell differentiation is associated with a decrease in system L transport capacity. EGF activates system L transport activity through a signaling mechanism involving protein kinase C, independent of cell differentiation state. Both cell differentiation and EGF regulation of system L activity occur via alteration of functional copies of the system L transporter. (J GASTROINTEST SURG 2002;6:410–417.)

KEY WORDS: Alanine, Caco-2, epidermal growth factor, differentiation, membrane transport

Intestinal amino acid absorption is a critical step for animals to obtain dietary amino acids, which are the essential building elements for protein synthesis and biological functions. Luminal amino acids are absorbed by enterocytes via discrete apical amino acid transport systems.[1] Migrating along the crypt-villus axis, the undifferentiated crypt cells differentiate into mature villous enterocytes. However, information is limited regarding the mechanism by which amino acid transport is regulated during the differentiation process. The human intestinal epithelial cell line Caco-2 undergoes spontaneous differentiation after attaining confluence in culture[2,3] and has been widely accepted as a useful in vitro model for small intestinal epithelial nutrition studies.[4,5] Certain orally delivered factors and luminal paracrines, such as epidermal growth factor (EGF), affect the luminal epithelium and regulate many biological functions in the small intestine.[6,7] Endogenous sources include secretions from the submaxillary gland and jejunal/ileal mucosa,[8] whereas exogenous sources include milk.[6,7,9] EGF has been shown to stimulate cell growth, proliferation, and differentiation in the epithelial cells. EGF is the main stimulator in promoting intestinal mucosal wound healing in mucosal injury.[10] EGF elicits its functions through binding to the same EGF receptor, a tyrosine kinase in the plasmic membrane, which triggers a series of intracellular cascades to exert its many biological activities.[6,7]

Previously we have demonstrated that EGF stimulated sodium-dependent system B alanine transport and sodium-independent system y^+ arginine transport across apical membrane in Caco-2 cells.[11,12] System L is a major Na^+-independent transport system for neutral amino acid transport in many tissues including intestine.[1] In this study we investigated the effect of cell

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (poster presentation).

From the Department of Surgery (M.P., W.W.S., A.M.K., C.M.L.), Pennsylvania State College of Medicine, Milton S. Hershey Medical Center, Hershey, Pennsylvania; and the Department of Physiology (B.R.S.), College of Medicine, University of Florida, Gainesville, Florida.
Reprint requests: Ming Pan, M.D., Ph.D., Department of Surgery, H149, Pennsylvania State College of Medicine, MC 850, Hershey, PA 17033.

Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(02)00004-5

differentiation and EGF on System L alanine transport activity in human Caco-2 cell membranes.

MATERIAL AND METHODS

Caco-2 Cell Cultures

The established human intestinal epithelial Caco-2 cell line was obtained from American Type Culture Collection (Rockville, Maryland) at passage 16. Cells were grown in a humidified incubator at 37° C in 10% CO_2/90% O_2. Cells were routinely grown in Dulbecco's modified eagle medium (DMEM) containing 4.5 g/L glucose, 0.584 g/L glutamine, 10% fetal bovine serum, 3.7% sodium bicarbonate, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1% nonessential amino acids. Caco-2 cells were passaged weekly after treatment with 0.05% trypsin and 0.02% ethylenediamine tetraacetic acid. Cells were reseeded at a density of 4.5×10^6 cells per 100 mm dish for future subculturing, or seeded in the six-well cluster Falcon tissue culture dishes at a density of 4×10^5 cells per 35 mm well for transport experiments. Cells (passage 19 to 40) were used for experiments. The day of seeding was designated as day 0. Cells reached confluence on postseeding days 5 and 6 and underwent spontaneous differentiation. Preconfluent day 3 cells were undifferentiated and postconfluent day 9 cells were differentiated cells, as determined by intestinal epithelial membrane enzyme markers such as alkaline phosphatase and sucrase, and so forth.[2–5] The growth medium was changed daily, and cultures were inspected daily using a phase-contrast microscope.

Cell Treatments

Cells were treated with various agents including EGF, cycloheximide (CHX), actinomycin D (Act-D), or chelerythrine chloride (CHE). To treat cells, growth medium was first replaced with serum-free medium (i.e., DMEM containing nonessential amino acids, penicillin, and streptomycin, but lacking fetal bovine serum) for 2 hours at 37° C. The cells were then exposed to each agent at the various times and concentrations described below. Pretreatment buffers were replenished every 6 hours. Caco-2 cells remained healthy (viability >99% by dye exclusion) during at least 48 hours of exposure to serum-free media. 12-O-tetradecanoyl-phorbol-13-acetate (TPA) was prepared from dimethylsulfoxide (Me_2SO) stocks, giving less than 0.5% Me_2SO in final media exposed to cells. This concentration of dimethylsulfoxide did not influence alanine uptake activity (28.8 ± 54 pmole/mg/minute control vs. 32 ± 4.6 pmole/mg/minute 0.5% Me_2SO, n = 6, P = NS).

L-Alanine Uptake Measurements

[^{3}H]-L-alanine (Amersham Co., Arlington Heights, Illinois) uptake across apical membrane was measured in cells ranging in age from 1 day post seeding (undifferentiated) through 16 days post seeding (differentiated). Cultures were confluent on about day 6. Studies designed to compare transport in cells 3 days post seeding to 9 days post seeding were conducted using cells started from the same seeding parent cells. Transport activity was measured at room temperature (23° C ± 1.0° C). After cells were pretreated with various agents (described above), cells were rinsed with "uptake buffer" (23° C) comprised of 137 mmol/L choline chloride (choline Cl), 10 mmol/L HEPES/Tris buffer (pH 7.4), 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, and 2.5 mmol/L $CaCl_2$. The uptake was initiated by adding 1 ml of this buffer also containing [3H]-L-alanine (2 μCi/ml, 1 μmol/L to 5 mmol/L). Culture dishes were continuously shaken by an orbital shaker (1 Hz) during the uptake period. Uptake was arrested by aspirating the uptake buffer and washing three times with ice-cold buffer lacking substrate. Radioactivity of isotope extracted from the cells with 1 ml 1N NaOH was neutralized with acetic acid, and then assayed by liquid scintillation spectrometry. Protein in the NaOH extract was measured using the Bio-Rad protein assay. Initial rates of transport activity were determined during the linear uptake period (2 minutes), with zero time points serving as blanks. Uptake rates are expressed as nanomoles of alanine per minute per milligram of cell protein.

Data Analysis

All experiments were conducted at least in triplicate (including the zero-time blanks), and all experiments were confirmed using at least two independently passaged generations of cells. Experimental means are reported ± standard error of the mean (SEM). Comparisons of means were made by analysis of variance with pairwise multiple comparisons by the Newman-Keuls method at $P < 0.05$. Transport kinetic parameters were obtained by fitting data to the Michaelis-Menten equation by linear or nonlinear regression analysis.

RESULTS

L-Alanine Transport Time Course

Uptake of [3H]-L-alanine was linear up to at least 10 minutes at L-alanine concentrations of 50 μmol/L or 5 mmol/L in 137 mmol/L choline Cl media (Fig. 1). Alanine uptake rates measured in media containing 137

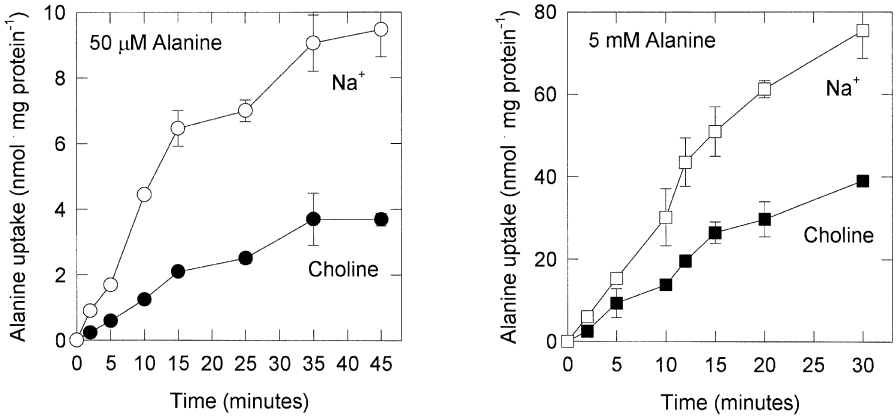


Fig. 1. Sodium-independent alanine uptake time course. Uptake of alanine (50 μmol/L and 5 mmol/L) was measured in choline chloride buffer in cells 3 days and 9 days after passaging.

mmol/L mannitol or 137 mmol/L chloride or gluconate salts of K^+ or Li^+ were not significantly different from rates with choline Cl ($P < 0.05$; data not shown). For all subsequent measurements, an uptake period of 0 to 2 minutes in choline Cl media was chosen.

Effect of pH on the L-Alanine Uptake

L-alanine (50 μmol/L) uptake was measured in choline Cl medium at pH 6.4, 7.4, and 8.4 using HEPES and Tris buffers. The alanine uptake was unaffected by buffer pH in either day 3 or day 9 cells. Subsequent experiments were conducted with uptake buffer at pH 7.4.

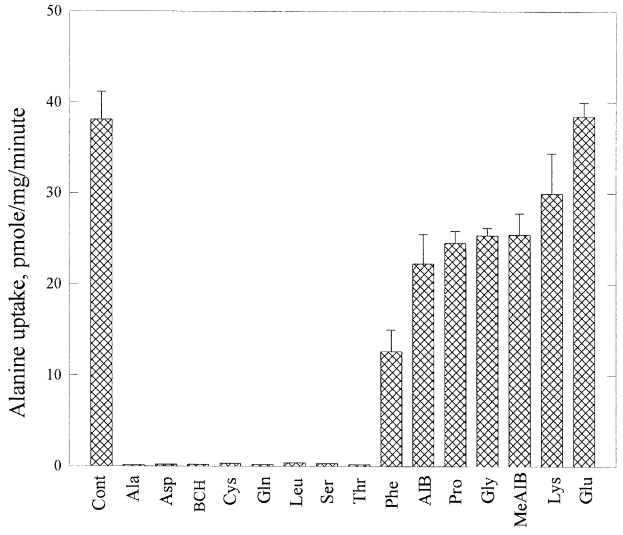


Fig. 2. Alanine uptake inhibition by amino acid analogues in day 3 cells. Inhibition of 50 μmol/L [^{3}H]-L-alanine uptake by 5 mmol/L unlabeled amino acids, or mannitol control, in choline media. Similar results were observed in day 9 cells. Inhibitor symbols: cont, control; Ala, alanine; Asp, asparagine; BCH, 2-aminobicycloheptane-2-carboxylic acid; Cys, cysteine; Leu, leucine; Ser, serine; Thr, threonine; Phe, phenylalanine; AIB, aminoisobutyric acid; Pro, proline; Gly, glycine; MeAIB, α-methyl aminoisobutyric acid; Lys, lysine; Glu, glucose.

Amino Acid Analogue Inhibition

[^{3}H]-L-alanine (50 μmol/L) transport was measured, in both day 3 and day 9 cells, in uptake media containing 5 mmol/L–unlabeled amino acid analogues (mannitol as control). Fig. 2 shows the relative pattern of inhibition in day 3 cells. The pattern was similar at both cell ages, but uptake rates were consistently greater in day 3 cells compared to day 9 cells (data not shown). Amino acids and analogues phenylalanine, leucine, 2-aminobicycloheptane-2-carboxylic acid (BCH, a specific System L substrate), and alanine strongly inhibited the alanine uptake, whereas α-methyl amino isobutyric acid (MeAIB, a specific System L substrate) and glycine were weak inhibitors. These inhibition patterns strongly resemble that of the System L. Amino acid transport System L is a ubiquitous sodium-independent neutral amino acid expressed in many tissues and cells. Lysine did not interact with this alanine uptake, which ruled out the possibility of the system $b^{0,+}$. Strong inhibition of alanine uptake by BCH ruled out system asc as the transport system.

Effect of Cell Age and Differentiation on System L Alanine Transport

Fig. 3 demonstrates that the System L alanine uptake rates decreased as the cells aged. This uptake was highest in undifferentiated Caco-2 cells and declined as the cells reached confluence (at about day 5 to 6) and became differentiated (≥day 9). In our previous studies we have demonstrated that the Caco-2 cell proliferation rate declined as cells underwent

differentiation.[13] The decrease in activity was coincident with the cell proliferation rates.

Sodium-Independent Alanine Transport Kinetics in Undifferentiated and Differentiated Cells

Uptake of alanine of various concentrations (1 μmol/L to 5 mmol/L) was measured in both day 3 and day 9 cells. There were nonsaturable passive diffusion and saturable carrier-mediated components in alanine uptake. For the nonsaturable component, the passive permeability coefficient (P) describing the relation $J = P \cdot [Ala]$ was calculated at 0.53 ± 0.08 μg protein^{-1} min^{-1} in both day 3 and day 9 cells. Nonlinear analysis of the saturable component demonstrated a single Na^+-independent alanine uptake system. The maximal capacity (V_{max}) decreased from V_{max} = 1.85 ± 0.25 nmole mg protein^{-1} min^{-1} in the undifferentiated (day 3) cells to V_{max} = 0.38 ± 0.02 nmole mg protein^{-1} min^{-1} in the differentiated (day 9) cells. However, the Na^+-independent alanine transport apparent affinity (K_m) was unaffected by cell ages (K_m = 1.10 ± 0.19 mmol/L alanine in day 3 cells vs. differentiated cells K_m = 1.02 ± 0.01 mmol/L alanine) (Fig. 4).

Effect of EGF on System L Alanine Uptake Activity

Uptake of alanine (50 μmol/L) was measured in the Caco-2 cells (day 3 and day 9) after cells have been incubated in EGF (0 to100 ng/ml) for various periods of time (minutes to 72 hours). Continued incubation (48 hours) of EGF (10 ng/ml) resulted in a 70% increase of alanine uptake in day 3 cells (Fig. 5). Similar stimulation was observed in day 9 cells (14.2 ± 2.1 pmole/mg/minute control vs. 23.3 ± 3.1 pmole/mg/minute EGF, n = 6, $P < 0.01$). The System L alanine uptake activity was not affected by EGF at the incubation time less than 30 hours. The System L activity was not increased by the pulse EGF treatment. Act-D (0.1 μmol/L), CHX (1 μmol/L), or the specific protein kinase C inhibitor CHE (6.6 μmol/L) in the incubation medium blocked the EGF-induced System L alanine uptake (Fig. 6).The protein content and cell numbers of the 48 hours of Act-D–, CHX– ,or CHE–treated cells were comparable to pretreatment levels. The viability of Act-D/CHX/CHE–treated cells was greater than 99%. Compared to the control group (only DMEM treatment), the Act-D/CHX/CHE–treated cells had 20% less protein and 40% less cells. The inhibitory effect of Act-D, CHX, or CHE on the System L alanine uptake was likely due to inhibiting new protein synthesis rather than a cytotoxic effect.

Effect of EGF on System L Alanine Transport Kinetics

Uptake of alanine at various concentrations (1 μmol/L to 5 mmol/L) was measured in both day 3 and day 9 cells after cells have been incubated in EGF (10 ng/ml) for 48 hours. EGF stimulated the system L V_{max} in both undifferentiated cells (2.9 ± 0.3 nmole protein^{-1} min^{-1} vs. 1.85 ± 0.25 nmole

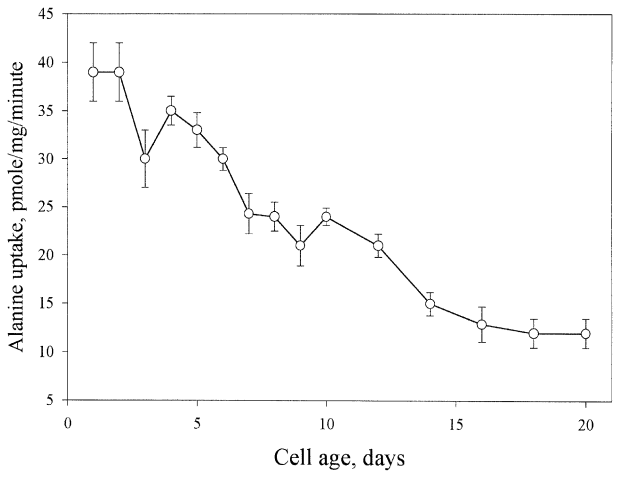


Fig. 3. Alanine initial uptake rates decreased with Caco-2 cell age. Alanine (50 μmol/L) initial rates of uptake were measured in Caco-2 cells of various ages ranging from 1 day through 14 days post passaging. A confluent monolayer was attained on day 6.

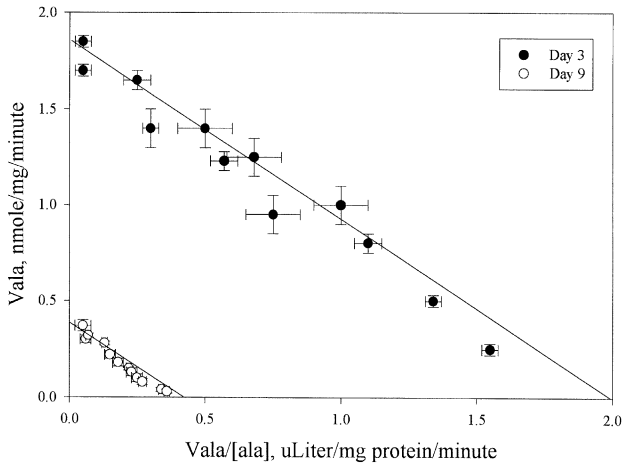


Fig. 4. System L kinetics in cells 3 days and in confluent cells 9 days post seeding (from the same passage). [^{3}H]-alanine (1 μmol/L to 5 mmol/L) uptake rates were measured and the data were corrected for the nonsaturable component that remained in the presence of 5 mmol/L unlabeled alanine. Regression analysis of the data gave one carrier-mediated component.

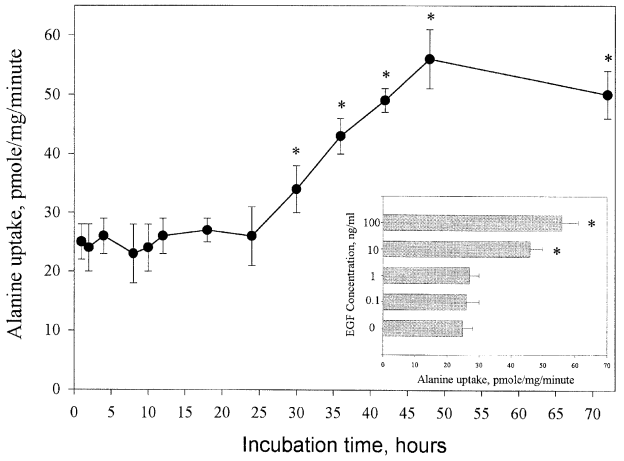


Fig. 5. The effect of EFG on system L alanine transport activity. Uptake of alanine (50 μmol/L) was measured in cells incubated in EGF (0 to 100 ng/ml) for various periods of time (minutes to 72 hours). *Inset*, The effect of EGF concentrations on system L alanine transport activity. Uptake of alanine (50 μmol/L) was measured in cells incubated in EGF (0 to 100 ng/ml) for 48 hours. *Mean values significantly different from control ($P < 0.05$).

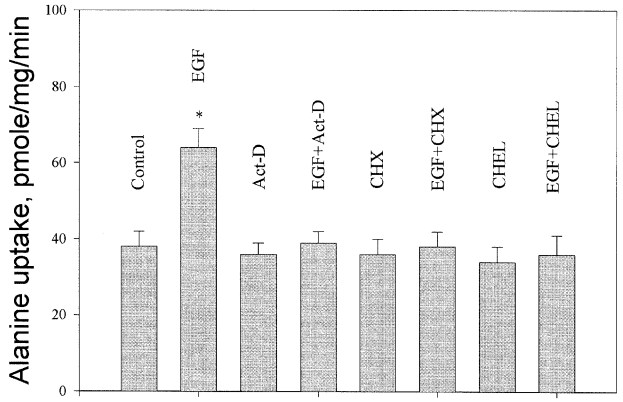


Fig. 6. The effect of Act-D, CHX, and CHEL on the EGF-stimulated system L activity. Uptake of alanine (50 μmol/L) was measured in cells incubated in EGF (10 ng/ml) ± Act-D (0.5 μmol/L), CHX (10 μmol/L), or CHE (6.6 μmol/L) for 48 hours. *Mean values significantly different from control ($P < 0.05$).

protein^{-1} min^{-1}, $P < 0.01$) and differentiated cells (0.48 ± 0.04 nmole protein^{-1} min^{-1} vs. 0.38 ± 0.02 nmole protein^{-1} min^{-1}, $P < 0.01$). However, the transport affinity K_m was not affected by EGF incubation in both day 3 and day 9 cells (Fig. 7).

Effect of Phorbol Ester on System L Alanine Transport

Uptake of alanine (50 μmol/L) was measured in the Caco-2 cells (day 3 and day 9) after cells had been incubated in 12-O-tetradecanoyl-phorbol-13-acetate (TPA; 1 nmol/L to 1 μmol/L) for various periods of time (minutes to 24 hours). Continued incubation (24 hours) of TPA (0.5 μmol/L) resulted in a 50% increase in alanine uptake (Fig. 8).

DISCUSSION

In the present study we characterized the sodium-independent alanine transport system in the apical membrane of Caco-2 cells and regulation of this transport system under the influence of cell differentiation and luminal growth factor–EGF.

Intestinal absorption of dietary amino acids is the first step in providing the essential building blocks of protein synthesis and associated biological components. Amino acids are absorbed by intestinal epithelium via discrete transport systems. The transport systems have been divided into the following three categories: (1) passive diffusion; (2) sodium-independent carrier-mediated transport; and (3) sodium-dependent secondary active transport. In our previous studies we have characterized the Na^+-dependent alanine System B transport systems and regulation.[11]

Amino acid transport systems are characterized on the basis of criteria pioneered by Christensen[14]—substrate selectivity, sodium dependency, pH sensitivity, amino acid analogue inhibition pattern, and kinetic analysis. The recent cloning of various amino acid transporters provides additional tools—for example, Northern blot analysis using cloned cDNA and Western blot analysis using specific antibody—in studying amino acids transport systems.[15–18]

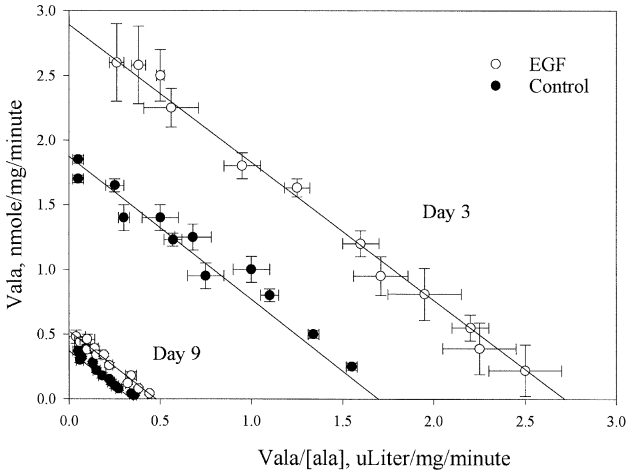


Fig. 7. Eadie-Hofstee plot of system L alanine uptake kinetics. System L alanine (1 μmol/L to 5 mmol/L) uptake was measured in cells (day 3 and day 9) incubated in medium ± EGF (10 ng/ml) for 48 hours.

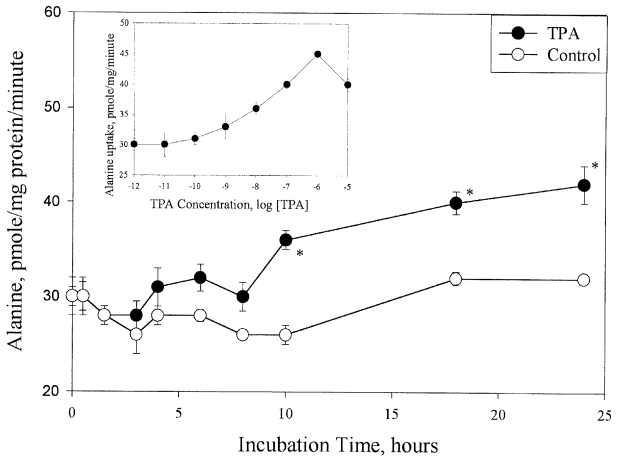


Fig. 8. Effect of phorbol ester on alanine transport activity. Uptake of alanine (50 μmol/L) was measured in cells incubated in 12-O-tetradecanoyl-13-acetate (TPA; 6.6 μmol/L) for up to 24 hours. *Inset*, the TPA dose-response curve. *Mean values significantly different from control ($P < 0.05$).

Caco-2 cells undergo spontaneous differentiation after attaining confluence, and the differentiated epithelium demonstrates characteristics unique to small intestinal epithelial cells. The Caco-2 cell model has been widely used as an in vitro small intestinal epithelial model in a variety of nutrient absorption studies, including amino acid absorption, in our laboratory.[4,5,11,12]

As shown in Fig. 3, there are two transport systems for the sodium-independent alanine uptake in Caco-2 cells: passive diffusion and a saturable transport system. The passive permeability coefficient for alanine was the same measured in both the day 3 and day 9 cells. This suggested that the Caco-2 cell development did not alter the membrane permeability to alanine. In other words, the membrane noncarrier passive diffusion rates for alanine were the same regardless of differentiation state. Of course, the contribution by passive diffusion varies with alanine concentrations such that at [alanine] = 50 μmol/L, the passive uptake contributes to alanine uptake was minimal (<1% total uptake), whereas at [alanine] = 5 mmol/L, passive diffusion contributes more than 90% to total alanine uptake.

The amino acid analogue inhibition pattern of sodium-independent alanine uptake was similar for both the day 3 and day 9 cells. Unlabeled alanine, asparagine, glutamine, BCH, cysteine, leucine, serine, threonine, and phenylalanine each strongly inhibited radiotracer alanine uptake, whereas AIB, glycine, and MeAIB partially inhibited alanine uptake (see Fig. 2). These inhibition patterns strikingly resemble that of System L, as expressed in a variety of cell types.[19] The lack of cationic amino acid interaction with alanine uptake operationally precludes a significant contribution by System $b^{0,+}$ in our Caco-2 system.[20] The strong inhibition by BCH rules out the other major sodium-independent pathway, system asc.[21] System L is a ubiquitous neutral amino acids transport system that is present in many tissues.[22] Despite its ubiquitous presence, System L has not been cloned from Caco-2 cells, thus impeding a more complete understanding of its regulation.

The System L alanine uptake in Caco-2 cells decreased as cells aged, suggesting that the System L transport activity is regulated by cell age and differentiation states. The same inhibition pattern of alanine transport observed in both day 3 (see Fig. 2) and day 9 cells suggests that the same System L exists in both states. As shown in Fig. 4, the System L activity changes between the two states was due to a decrease of V_{max} in differentiated state (1.85 ± 0.25 nmole mg protein^{-1} min^{-1} in day 3 cells vs. 0.38 ± 0.02 nmole mg protein^{-1} min^{-1} in day 9 cells), whereas the transport affinity K_m was virtually unchanged. These kinetic parameters strongly indicate that the transport activity difference between day 3 and day 9 cells represents the changes in the numbers of functional transport units that are present at the membrane. Further studies, such as Western blotting with the use of specific anti–System L antibody when it became available, will be needed.

Peptide growth factor EGF, which is normally present in the intestinal lumen and in circulation, has been shown to regulate epithelial cell growth, proliferation, and differentiation.[6,7] EGF has also been shown to promote intestinal protein synthesis and mucosal repair.[21] Intestinal luminal EGF can either from endogenous sources such as submandibular glands and intestinal jejunal mucosa[6,7,9] or an exogenous source such as milk.[9] EGF elicits its functions through binding to the same EGF receptor, a tyrosine kinase in the plasmic membrane, which regulates many biological activities. The EGF receptor then activates phospholipase, mitogen-activated protein kinase, lipoprotein I, c-erbB-2, and phosphoinositol-3 kinase.[23–26] In our previous studies we have shown that EGF stimulates sodium-dependent alanine and sodium-independent arginine uptake via intracellular protein kinase C activation.[11,12]

EGF elicits its biological activities through two classes of mechanisms: an acute phase (in minutes) and a chronic phase (hours). The acute phase involves intracellular phosphorylation to elicit rapid responses. On the other hand, the chronic phase normally involves intracellular cascade and intracellular protein synthesis to elicit slow but sustained responses.[27] During the continuous incubation of EGF, the incubation medium was changed every 6 hours to ensure

a consistent EGF exposure and minimize the possibility that involvement of the paracrine effect may be associated with EGF exposure. As shown in Fig. 5, prolonged EGF exposure stimulated System L activity in a time- and dose-dependent manner in day 3 cells. Similar results were observed in day 9 Caco-2 cells. More than 30 hours of continuous incubation was required for EGF to elicit the stimulatory effect, suggesting that EGF-induced alanine transport stimulation participates in the chronic phase of EGF activity rather than triggering an acute effect.

Act-D or CHX in the incubation medium each blocked the EGF-induced alanine uptake, indicating the possible involvement of transcription and de novo protein synthesis. The protein kinase C inhibitor CHEL blocked the EGF-induced alanine uptake and the direct activation of protein kinase C by phorbol ester–stimulated alanine uptake, thereby demonstrating the involvement of protein kinase C in signaling events associated with System L induction in Caco-2 cells. Kinetic analyses of System L activity showed that EGF stimulated the transport maximal capacity V_{max} without affecting the apparent K_m. These data indicate that EGF stimulates alanine uptake by increasing functional copies of System L transport units rather than modifying transport affinity. Since a System L probe is currently not available, it is unclear whether the observed increase in transport activity V_{max} reflects de novo protein synthesis of a transporter protein itself or another regulatory protein.

In summary, sodium-independent alanine uptake in Caco-2 apical membrane occurs via saturable transport System L plus passive diffusion in both the undifferentiated and differentiated states. Furthermore, cell differentiation results in a decrease in System L activity. EGF stimulates the System L alanine membrane transport capacity in both undifferentiated and differentiated states, likely by increasing the transporter functional units of the copies rather than modifying transporter affinity. Finally, signaling pathways leading to activation of System L likely involve protein kinase C.

REFERENCES

1. Stevens BR. Amino acid transport in intestine. In Kilberg MS, Häussinger D, eds. Mammalian Amino Acid Transport Mechanisms and Control. New York: Plenum, 1992, pp 149–163.
2. Hidalgo IJ, Raub TJ, Borchardt RT. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 1989;96:736–749.
3. Mohrmann IM, Mohrmann J, Biber H, Murer H. Sodium-dependent transport of P_i by an established intestinal epithelial cell line (Caco-2). Am J Physiol 1986;250:323–330.
4. Blais A, Bissonnette P, Berteloot A. Common characteristics for Na^+-dependent sugar transport in Caco-2 cells and human fetal colon. J Membrane Biol 1987;99:113–125.
5. Brandsch M, Miyamoto Y, Ganapathy Y, Leibach FH. Expression and protein kinase C-dependent regulation of peptide/H^+ cotransport system in the Caco-2 human colon carcinoma cell line. Biochem J 1994;299:253–260.
6. Carpenter G, Cohen S. Epidermal growth factor. J Biol Chem 1990;265:7709–7712.
7. Hernandez-Sotomayor SMT, Carpenter G. Epidermal growth factor receptor: Elements of intracellular communication. J Membrane Biol 1992;128:81–89.
8. Thompson JF. Specific receptors for epidermal growth factor in rat intestinal microvillus membranes. Am J Physiol 1988;254:429–435.
9. Britton JR, George-Nascimento C, Udall JN, Koldovsky O. Minimal hydrolysis of epidermal growth factor by gastric fluid of preterm infants. Gut 1989;30:327–332.
10. Podolsky DK. Healing the epithelium: Solving the problem from two sides. J Gastroenterol 1997;32:122–126.
11. Pan M, Stevens BR. Differentiation- and protein kinase C-dependent regulation of alanine transport via system B. J Biol Chem 1995;270:3582–3587.
12. Pan M, Stevens BR. Protein kinase C-dependent regulation of L-arginine transport activity in Caco-2 intestinal cells. Biochim Biophys Acta 1995;1239:27–32.
13. Pan M, Malandro M, Stevens BR. Regulation of system y+ arginine transport capacity in differentiating human intestinal Caco-2 cells. Am J Physiol 1995;268:578–585.
14. Christensen HN. Role of amino acid transport and countertransport in nutrition and metabolism. Physiol Rev 1990; 70:43–77.
15. Wang H, Kavanaugh MP, North RA, Kabat D. Cell-surface receptor for ecotopic murine retroviruses is a basic amino-acid transporter. Nature 1991;352:729–731.
16. Rajan DP, Huang W, Kekuda R, George RL, Wang J, Conway SJ, Devoe LD, Leibach FH, Prasad PD, Ganapathy V. Differential influence of the 4F2 heavy chain and the protein related to b(0,+) amino acid transport on substrate affinity of the heteromeric b(0,+) amino acid transporter. J Biol Chem 2000;275:14331–4335.
17. Sugawara M, Nakanishi T, Fei YJ, Huang W, Ganapathy ME, Leibach FH, Ganapathy V. Cloning of an amino acid transporter with functional characteristics and tissue expression pattern identical to that of system A. J Biol Chem 2000; 275:16473–16477.
18. Gu S, Roderick HL, Camacho P, Jiang JX. Identification and characterization of an amino acid transporter expressed differentially in liver. Proc Natl Acad Sci U S A 2000;97:3230–3235.
19. Stevens BR, Ross HJ, Wright EM. Multiple transport pathways for neutral amino acids in rabbit jejunal brush border vesicles. J Membrane Biol 1982;66:213–225.
20. Van Winkle LJ, Campione AL, Gorman JM. Na+-independent transport of basic and zwitterionic amino acids in mouse blastocysts by a shared system and by processes which distinguish between these substrates. J Biol Chem 1988;263:3150–3163.
21. Kilberg MS, Handlogten ME, Christensen HN. Characteristics of an amino acid transport system in rat liver for glutamine, asparagine, histidine, and closely related analogs. J Biol Chem 1980;255:4011–4019.
22. Betran J, Testar X, Zorzano A, Palacin M. A new age for mammalian plasma membrane amino acid transporters. Cell Physiol Biochem 1994;4:217–241.
23. Margolis B, Rhee SG, Felder S, Mervic M, Lyall R, Levitzki A, Ullrich A, Zilberstein A, Schlessinger J. EGF induces tyrosine phosphorylation of phospholipase C-II: A potential mechanism for EGF receptor signaling. Cell 1989;57:1101–1107.

24. Ellis C, Moran M, McCormick F, Pawson T. Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. Nature 1990;343:377–381.
25. Akiyama T, SaitoT, Ogawara H, Toyoshima K, Yamamoto T. Tumor promoter and epidermal growth factor stimulate phosphorylation of the c-erbB-2 gene product in MKN-7 human adenocarcinoma cells. Mol Cell Biol 1988;8:1019–1026.
26. Fain JN. Regulation of phosphoinositide-specific phospholipase C. Biochim Biophys Acta 1990;1053:81–88.
27. Carpenter G, Soler C, Baulida J, Beguinot L, Sorkin A. Interaction of signaling and trafficking proteins with the carboxyterminus of the epidermal growth factor receptor. Ann N Y Acad Sci 1995;766:44–51.

Differential Adrenergic Response to Extrinsic Denervation in Canine Longitudinal Jejunal and Ileal Smooth Muscle

Nicholas J. Zyromski, M.D., Judy A. Duenes, B.S., Michael L. Kendrick, M.D., Karen D. Libsch, M.D., Roland Seiler, B.S., Toshiyuki Tanaka, M.D., Michael G. Sarr, M.D.

Early postoperative complications after small bowel transplantation (SBT) are likely mediated, at least in part, by dysmotility caused by the obligate disruption of extrinsic and enteric nerves in the graft. Adrenergic hypersensitivity of gut smooth muscle has been observed in some (but not all) segments of intestine in various experimental models of SBT, highlighting regional and species variability in response to denervation. Little is known about changes in canine longitudinal muscle after extrinsic denervation. Six dogs each underwent either complete extrinsic denervation of the jejunoileum or a control operation (transection and reanastomosis of the proximal jejunum and distal ileum). In vitro contractile response of longitudinal muscle strips to norepinephrine was evaluated at the time of the operation, and 2 weeks and 8 weeks later. After extrinsic denervation, the jejunal response to norepinephrine was preserved at all time points; however, the ileum displayed a decreased sensitivity to norepinephrine, an effect unmasked after intramural neural blockade with tetrodotoxin. These data support a potential for neurally mediated dysmotility after SBT and reinforce the differences in responses to extrinsic denervation between species, as well as differences within different regions and between anatomic segments of small intestine in the same species. (J GASTROINTEST SURG 2002;6:418–425.)

KEY WORDS: Motility, smooth muscle, denervation, extrinsic denervation, adrenergic innervation, adrenergic denervation, small bowel transplantation

Small bowel transplantation (SBT) has become a clinical reality, offering lifesaving therapy to select patients with short bowel syndrome.[1] Recent advances in immunosuppression have dramatically improved long-term clinical outcomes; however, diarrhea and intolerance of enteral feeding remain troublesome complications in the early postoperative period.[2] Although these complications are poorly understood and likely multifactorial, both clinical and experimental studies have demonstrated alterations in motility after SBT that likely play a primary role in their genesis.[3–8] Attempts to elucidate the mechanism(s) of these complications have been hampered by the complexity of the enteric nervous system, as well as by the numerous regional and species differences in intestinal contractility. In addition to having different structural and contractile characteristics, the different segments and layers of the small bowel (i.e., jejunum vs. ileum; circular smooth muscle vs. longitudinal smooth muscle) receive variable extrinsic and enteric neural input and may respond differently to denervation.[9–13] Further confounding this issue is the observation that the same neurotransmitter may cause a markedly different response in various regions of the small bowel. The adrenergic neurotransmitter norepinephrine (NE), for example, generally causes relaxation of enteric smooth musculature and inhibition of contractile activity. In the canine circular and equine longitudinal smooth muscle of the small bowel, however, NE acts as an excitatory agent, causing a predominantly contractile response.[14,15]

Presented in part at the Ross Residents and Fellows Research Conference and the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (poster presentation); and published as an abstract in *Gastroenterology* 120:A351, 2001.
From the Gastroenterology Research Unit and Department of Surgery (N.J.Z., J.A.D., M.L.K., K.D.L., R.S., T.T., M.G.S.), Mayo Clinic, Rochester, Minnesota.
Supported by grants from the National Institutes of Health (NIH DK39337 and NIH DK07198) and the Mayo Foundation.
Reprints not available. Address correspondence to: Michael G. Sarr, M.D., Professor of Surgery, Chair, Division of General and Gastroenterologic Surgery, Gastroenterology Research Unit (AL-2-435), Mayo Clinic, 200 First Street SW, Rochester, MN 55905.

 Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(02)00006-9

The role of extrinsic neural input in modulating intestinal contractility is also an important consideration because SBT necessitates extrinsic denervation. Animal models of extrinsic denervation and SBT have revealed a marked adrenergic hypersensitivity in the canine circular smooth muscle of the jejunum and ileum,[14,16] as well as the circular and longitudinal smooth muscle of the rat ileum, but not in the rat jejunum.[17–20] The effects of extrinsic denervation on contractile activity of longitudinal smooth muscle in the canine small intestine are unknown.

These experiments were therefore designed to evaluate changes in adrenergic sensitivity in longitudinal smooth muscle of the canine jejunum and ileum, in a large animal model of SBT, focusing specifically on the effects of extrinsic denervation. Our hypothesis was that extrinsic denervation would cause an increase in adrenergic sensitivity in the longitudinal musculature of the canine small bowel similar to that observed in canine circular smooth muscle.

METHODS

Surgical procedures, subsequent care of animals, and conduct of experiments received approval from and were carried out according to criteria set forth by the Animal Care Committee of the Mayo Clinic Foundation and in accordance with guidelines of the National Institutes of Health and the United States Public Health Service policy on the humane use and care of laboratory animals.

Preparation of Animals

Twelve healthy female mongrel dogs (15 to 24 kg) were randomized into two groups. A control group of six dogs underwent celiotomy with selective transection and end-to-end reanastomosis of the proximal jejunum and distal ileum (without disruption of the mesentery) to control for the effects of anesthesia, celiotomy, and disruption of enteric neural continuity to the jejunoileum. The other six dogs underwent complete extrinsic denervation of the jejunoileum by a previously described model of in situ neural isolation of the entire jejunoileum.[3] Briefly, after induction of anesthesia with 12 mg/kg intravenous methohexital sodium and maintenance of anesthesia with inhaled 1.5% isoflurane, the peritoneum was accessed via a midline celiotomy. The small intestine was transected 3 cm distal to the ligament of Treitz and 3 cm proximal to the ileocecal junction, disrupting continuity of the enteric nervous system and ensuring division of any extrinsic nerve fibers traveling within the wall of the bowel. The jejunoileal mesentery was then divided in radial fashion from the sites of intestinal transection back to the base of the small bowel mesentery at the origin of the superior mesenteric vessels. All extrinsic nerves and lymphatics traveling to the jejunoileum were carefully divided. In addition, the superior mesenteric artery and vein were stripped of investing adventitia for a length of 2 cm, including all perivascular neural tissue, under optical magnification. Enteric continuity was reestablished by hand-sewn, end-to-end anastomoses proximally and distally. This model has been validated to ensure complete extrinsic denervation of the jejunoileum[3] while specifically avoiding confounding factors that may affect contractile activity such as ischemia/reperfusion and immunologic effects (which would be necessitated by models of transplantation).

Full-thickness samples of proximal jejunum and distal ileum were obtained for study at the time of the initial operation (0 weeks), and at 2 weeks and 8 weeks postoperatively. At 0 weeks and 8 weeks, 2 cm segments of bowel were excised; 2 weeks after the initial operation, a minilaparotomy was performed and noncircumferential full-thickness jejunal and ileal biopsy samples were excised from the antimesenteric side of the bowel. Samples taken at the 2-week and 8-week time points were excised 10 cm (distally in jejunum and proximally in ileum) from the points of anastomosis or prior biopsy, respectively.

Experimental Protocol

Tissue biopsy samples were placed immediately in preoxygenated, iced Krebs buffer (in mEq/L or mmol/L) as follows: NaCl, 118.3; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; calcium disodium edetate, 0.26; and glucose, 11.1 and transported to the laboratory. Working in a Petri dish with oxygenated Krebs buffer, the mucosa was excised sharply, and 2×10 mm full-thickness muscle strips were cut in the orientation of the longitudinal muscle. The circular smooth muscle was deliberately left intact to ensure preservation of the myenteric nerve plexus. Muscle strips were suspended in separate 10 ml glass tissue chambers filled with Krebs buffer and maintained at 37° C with a mixture of 95% O_2 and 5% CO_2 bubbled continuously throughout the experiments. A 5-0 suture was used to attach muscle strips to a fixed hook at the bottom and to a force transducer (Kulite Semiconductor Products, Inc., Leonia, New Jersey) at the top of the chamber. This setup allowed isometric measurement of contractile force in the longitudinal direction. Because of the relatively small amount of circular muscle and the fact that it contracts perpendicularly to the longitudinal orientation of the muscle strips, contribution of circular

muscle contractions to the measured force should be negligible. Buffer was exchanged three times at 15-minute intervals, and the muscle strips were then allowed to equilibrate for 90 minutes. Strips were then incrementally stretched to optimal length (defined as the point past which further stretching produced no additional increase in amplitude or frequency of contraction) and maintained at this length for the duration of the experiments.

Adrenergic contractile response was evaluated by the cumulative addition of increasing doses of NE (10^{-8} to 10^{-6} mol/L). To eliminate enteric neural input and to test the effect of NE directly on jejunal and ileal smooth muscle, baseline conditions were reestablished, and the dose-response curves were reconstructed in the presence of 10^{-6} mol/L tetrodotoxin (TTX), a sodium channel blocker that inhibited any effects mediated by enteric (intrinsic) nerves within the muscle strip (i.e., myenteric neural plexus). Pilot studies demonstrated that this dosage of TTX inhibits the contractile response of longitudinal muscle strips to electric field stimulation, effects known to be mediated by the intramural nerves. At the conclusion of the experiments, muscle strips were blotted dry, and individual weights were recorded for standardization. All chemicals were obtained from Sigma Corporation, St. Louis, MO.

Data Analysis/Statistics

Contractile activity from the strain gauges was recorded simultaneously on a computerized data collection system (AcqKnowledge 3.2, BIOPAC Systems, Inc., Goleta, CA) and a Grass polygraph recorder (Grass Instruments, Quincy, MA) for visual inspection. Total area under the contractile curve was measured using specifically modified software and expressed in grams of force over 5 minutes, standardized per milligram of tissue wet weight. For statistical comparison of the effects of NE between groups, the dosage of NE needed to achieve 50% of baseline contractile activity (ED_{50}) was calculated. Simple and repeated-measures analysis of variance and paired or nonpaired Student's t test (with Bonferroni correction) were applied where appropriate when comparing changes within and between groups. $P < 0.05$ was accepted as statistically significant. Data are presented as mean ± standard error of the mean unless otherwise specified.

RESULTS

General Health

Dogs remained healthy and maintained good appetites throughout the course of the experiments. During the first 2 weeks after surgery, animals lost an average of 4% to 6% of their body weight; most dogs regained the weight thereafter. During the immediate postoperative period, animals that had undergone extrinsic denervation had explosive, watery diarrhea, which gradually resolved to loosely formed stools over the ensuing 2 to 3 weeks. At the time of both minilaparotomy and death (2 weeks and 8 weeks later, respectively), the bowels of all dogs appeared grossly normal with no evidence of functional or mechanical obstruction.

Baseline Contractile Activity

Jejunum. Jejunal muscle strips demonstrated regular spontaneous contractile activity at a frequency of 9.3 ± 0.2 contractions per minute (Fig. 1, *A*). No differences in spontaneous contractile activity were observed between time points (0, 2, or 8 weeks) in either the control ($P = 0.1$) or extrinsically denervated ($P = 0.5$) groups (Table 1). Extrinsic denervation did not alter baseline contractile activity when compared to values in time-matched control dogs ($P = 0.9$). Baseline contractile activity of the jejunum was subjectively noted to be more labile than that of the ileum, varying both in frequency and amplitude of contractions; indeed there was significantly less spontaneous contractile activity in the jejunum compared to the ileum ($P = 0.006$).

Ileum. Ileal muscle strips also demonstrated regular spontaneous contractile activity at a lower frequency than that used for the jejunal strips (8.5 ± 0.2 contractions/min). In contrast to the jejunal strips, contractile activity in the ileum was more consistent,

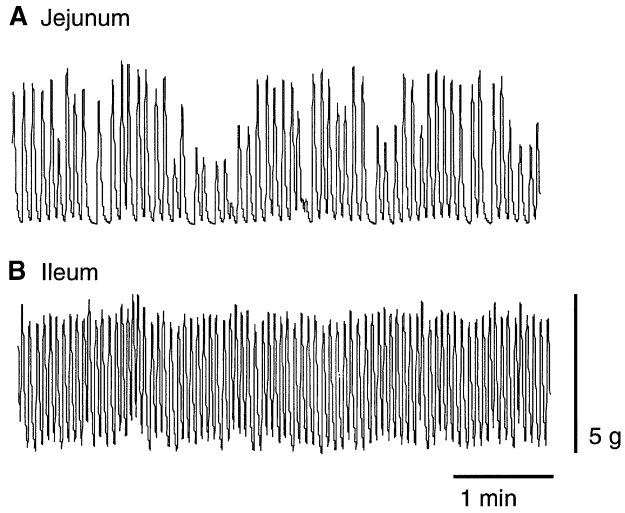


Fig. 1. Spontaneous contractile patterns of canine longitudinal smooth muscle strips from (**A**) jejunum and (**B**) ileum.

Table 1. Effect of TTX (10^{-6} mol/L) on spontaneous contractile activity of canine longitudinal muscle strips in vitro

Site	Time postop (wk)	Control		Extrinsically denervated	
		No TTX	TTX	No TTX	TTX
Jejunum	0	9.0 ± 1.9	4.5 ± 1.3*	10.0 ± 1.7	6.7 ± 1.5*
	2	8.5 ± 1.7	5.4 ± 1.0*	9.5 ± 1.9	6.7 ± 0.9*
	8	15.6 ± 3.8	8.6 ± 1.3	12.1 ± 1.6	7.5 ± 1.0*
Ileum	0	12.9 ± 1.7	8.9 ± 1.8*	14.9 ± 2.0	10.1 ± 0.9*
	2	17.5 ± 1.6	11.9 ± 1.5*	14.2 ± 1.4	9.3 ± 1.5*
	8	18.7 ± 2.2	12.0 ± 1.9*	12.0 ± 1.9	8.8 ± 1.5*

Values are mean ± SEM mg force/5 min/mg tissue weight; n = 6 dogs per group.
*Differs from No TTX group, $P < 0.01$. No statistical difference was seen at any time point within anatomic segments (jejunum and ileum) or between control and denervated groups.

with little variation in contractile amplitude (Fig. 1, *B*). Similar to what was observed in the jejunum, no differences in spontaneous contractile activity were observed at any time point (0, 2, or 8 weeks) within either the control ($P = 0.1$) or extrinsically denervated ($P = 0.5$) groups, and extrinsic denervation did not alter spontaneous contractile activity when compared to values in time-matched control dogs ($P = 0.1$) (see Table 1).

Effect of Tetrodotoxin on Basal Contractile Activity

Jejunum. Incubation of muscle strips with TTX (10^{-6} mol/L) produced a decrease in spontaneous contractile activity in both control ($P = 0.01$) and denervated ($P = 0.004$) dogs (see Table 1). Some muscle strips also had a decrease in basal tonic force after incubation with TTX. There were no differences in the degree of TTX-mediated decrease in contractile activity at any time point either in control ($P = 0.1$) or denervated ($P = 0.09$) dogs.

Ileum. TTX also caused a decrease in ileal spontaneous contractile activity in the control ($P = 0.008$) and denervated ($P = 0.002$) groups. As in the jejunum, TTX caused a decrease in basal tonic force in some ileal muscle strips. No differences in degree of TTX-mediated decreased contractile activity were observed between time points in either the control ($P = 0.2$) or denervated ($P = 0.8$) groups (see Fig. 2).

Effect of Norepinephrine

Jejunum. NE produced a dose-dependent decrease in spontaneous contractile activity and at higher doses a relaxation of basal force in all muscle strips (Fig. 3). There were no differences in the ED_{50} of NE at any of the three time points in the control group, either in the absence ($P = 0.2$) or presence ($P = 0.3$) of TTX (Table 2). Extrinsic denervation did not alter the ED_{50} of NE; no differences were seen between control and extrinsically denervated groups at any time point either in the absence ($P = 0.8$) or presence ($P = 0.5$) of TTX. Within the denervated group, the ED_{50} of NE did not differ at any of the three time points either in the absence ($P = 0.6$) or presence ($P = 0.9$) of TTX.

Ileum. Similar to jejunal longitudinal smooth muscle, NE caused a dose-dependent decrease in contractile activity in all ileal muscle strips at all time points, with higher doses causing a decrease in basal tonic force. There were no differences in the ED_{50} of NE at any time point within the control group either in the absence ($P = 0.4$) or presence ($P = 0.1$) of TTX. No differences in the ED_{50} were observed between the control and extrinsically denervated groups either in the absence ($P = 0.1$) or presence ($P = 0.6$) of TTX. Similarly, there were no differences in the effect of NE within the denervated group at any time point in the absence of TTX ($P = 0.3$). In contrast to jejunal longitudinal smooth muscle, however, the addition of TTX

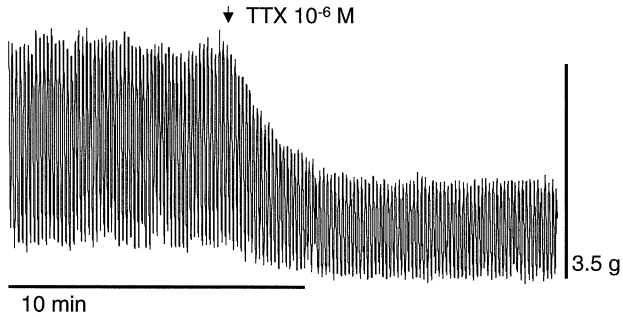


Fig. 2. Effect of TTX (10^{-6} mol/L) on spontaneous contractile activity of canine longitudinal ileal smooth muscle. TTX caused a similar effect in the jejunum.

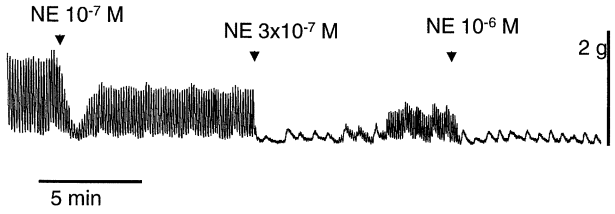


Fig. 3. Effect of increasing doses of norepinephrine (*arrows*) on contractile activity of canine longitudinal ileal smooth muscle. A similar effect was observed in the jejunum.

in the extrinsically denervated group caused a significant shift to the right of the dose-response curves ($P = 0.005$; Fig. 4); in other words, the addition of TTX revealed a decrease in tissue sensitivity to NE.

DISCUSSION

Our study showed that spontaneous contractile activity of the jejunal longitudinal muscle layer was qualitatively and quantitatively different from that of the ileum, although extrinsic denervation did not alter spontaneous contractile activity in either region. In both the jejunum and ileum, TTX decreased spontaneous contractile activity, and the adrenergic agonist NE caused a dose-dependent decrease in contractile activity. Perhaps the most interesting finding contradicted our initial hypothesis: extrinsic denervation did not cause adrenergic hypersensitivity in the longitudinal smooth muscle of the canine small intestine. The adrenergic effect was preserved unchanged in the longitudinal jejunum and, surprisingly, extrinsic denervation caused a decreased sensitivity to the adrenergic agonist NE in the ileal longitudinal smooth muscle when enteric nerves were blocked with TTX.

Early complications after SBT are likely caused by a number of different factors including graft ischemia-reperfusion injury, disruption of extrinsic and enteric neural continuity, and immunologic modulation. Our experimental preparation was designed to directly investigate the effects of extrinsic denervation while specifically avoiding these other confounding factors. Both clinical and animal models of SBT and extrinsic denervation have demonstrated that transection of extrinsic nerves and disruption of enteric neural continuity are at least partially responsible for dysmotility.[3–5] Indeed, our subjective observation of severe diarrhea in the group of dogs undergoing extrinsic denervation (compared to control dogs) supports this hypothesis.

Both extrinsic denervation and SBT have been shown experimentally to alter a number of small bowel neuromediators, including vasoactive intestinal polypeptide, substance P, neuropeptide Y, nitric oxide, and cholinesterase activity.[21–26] Several of our previous studies have shown a marked adrenergic hypersensitivity both in a rat model of isogenic SBT and in a canine model of extrinsic denervation.[14–20] We hypothesized that the adrenergic changes mediated by extrinsic denervation may contribute to the altered motility seen after SBT. Attempts to dissect the mechanism(s) of altered small bowel motility are complicated by differences between species, as well as by the known regional variability of small bowel structure, neurotransmitter and receptor population, and individual neurotransmitter function within the same species. Adrenergic innervation is no exception. Extrinsic innervation provides all of the adrenergic neural input to the small bowel; complete extrinsic denervation of the gut histologically, and functionally eliminates adrenergic nerves in the gut.[3,27–29] Except in sphincteric areas, where they are excitatory, adrenergic agonists generally act as inhibitory/relaxing agents throughout the gastrointestinal tract. Notable exceptions include the canine jejunal and ileal circular smooth muscle and equine longitudinal ileum, where adrenergic agonists cause a predominantly excitatory

Table 2. Effect of NE on canine longitudinal jejunal and ileal smooth muscle in the absence and presence of TTX (10^{-6} mol/L)

Site	Time postop (wk)	Control		Extrinsically denervated	
		No TTX	TTX	No TTX	TTX
Jejunum	0	7.1 ± 0.1	7.1 ± 0.1	6.9 ± 0.1	7.0 ± 0.1
	2	6.8 ± 0.2	6.8 ± 0.1	7.1 ± 0.1	6.8 ± 0.1
	8	6.9 ± 0.1	6.7 ± 0.4	7.1 ± 0.1	6.6 ± 0.2
Ileum	0	6.8 ± 0.1	6.5 ± 0.1	6.8 ± 0.1	6.6 ± 0.1*
	2	7.0 ± 0.1	6.9 ± 0.2	7.1 ± 0.1	6.7 ± 0.1*
	8	6.8 ± 0.1	6.8 ± 0.1	7.0 ± 0.2	6.7 ± 0.2*

Values are ED_{50} of NE, mean ± SEM; n = 6 dogs per group.
*Differs from No TTX group, $P < 0.05$.

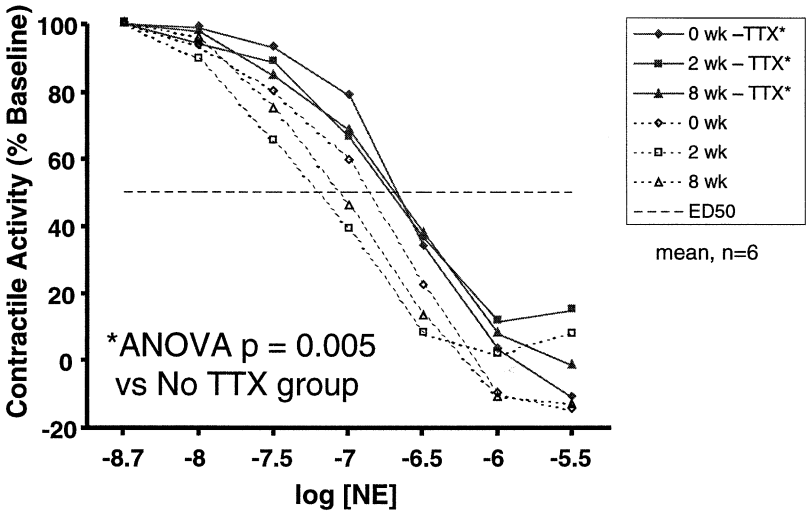


Fig. 4. Dose-response curves to NE in the longitudinal ileum of extrinsically denervated dogs without (*hatched lines*) and with (*solid lines*) TTX (10^{-6} mol/L). Mean of 6, SEM all < 15%; variance omitted for clarity.

effect.[14–16] Similarly, the population of adrenergic receptors is variable between species and in different regions of the small bowel. In a number of species (including dogs), alpha-adrenergic receptors are primarily located in presynaptic neurons of the enteric nervous system where they modulate release of acetylcholine.[30,31] Beta-adrenergic receptors are principally located on the smooth muscle cell membrane and cause relaxation of the smooth muscle directly via a G-protein/adenylyl cyclase–mediated second-messenger cascade.[30,31] Some evidence supports the location of presynaptic, myenteric beta-adrenergic receptors in the dog; however, the functional significance of these receptors is unclear.[32]

Given the structural and functional differences in various smooth muscle layers and regions of the small bowel, it was not surprising to find that baseline contractile activity in the longitudinal smooth muscle of the jejunum and ileum were qualitatively and quantitatively different. Although the frequency of spontaneous contractions was less in the ileum, contractile activity (measured as area under the contractile curve) was greater, and the contractile pattern and amplitude of contractions were generally more regular. In contrast, contractions in the longitudinal jejunum were more irregular and more labile (taking longer to recover after incubation with neuromediators or after washing the muscle strips). Extrinsic denervation did not alter the pattern or quantification of basal contractile activity in either the jejunum or ileum. Extrinsic denervation has previously been shown to decrease basal contractile activity in canine circular jejunum[33] but did not alter baseline contractile activity in the canine ileal circular smooth muscle.[16] These findings exemplify the differential response of various intestinal segments to extrinsic denervation.

Exogenous NE dose dependently decreased contractile activity in longitudinal muscle of both the jejunum and ileum. Although this is in accordance with adrenergic effects in many other areas of the gastrointestinal tract, it is in striking contrast to the adjacent circular muscle layers of the dog jejunum and ileum, where exogenous NE had an overall procontractile effect.[14,16] Longitudinal and circular muscle layers act in concert, and when circular muscle contracts, longitudinal muscle elongates (relaxes).[34] Although the mechanisms of longitudinal and circular synergistic interaction remain poorly understood and may well be mediated by different neurotransmitters, it is interesting to speculate that the variable response of circular and longitudinal muscle layers to NE may be secondary to differing populations of adrenergic receptors on the different muscle layers. Indeed Coupar and Liu,[10] in a unique experimental preparation designed to concurrently measure longitudinal and circular muscle contractions in an in situ segment of small bowel, demonstrated that alpha-adrenergic antagonists blocked cholinergic contractions induced by electrical field stimulation to much different degrees in circular and longitudinal smooth muscle, suggesting that variable populations of adrenergic receptors on circular and longitudinal smooth muscle layers translate to functional differences in contractile effect.

In many species, including the dog, presynaptic alpha-adrenergic receptors modulate release of acetylcholine from enteric nerves.[30] Thus, to evaluate

the effects of NE directly on the longitudinal smooth muscle, we used the sodium channel nerve blocker TTX to eliminate enteric neural input. Incubation of muscle strips with TTX decreased spontaneous contractile activity in both jejunum and ileum, which is a particularly interesting finding when contrasted to our previous study of canine jejunal and ileal circular smooth muscle, where enteric neural blockade by TTX caused an increase in basal contractile activity.[14,16] The fact that this TTX-mediated effect was not altered by extrinsic denervation suggests an alteration of tonic enteric neural input (as opposed to an alteration in tonic extrinsic neural input), and again highlights the differences in enteric input in adjacent layers of smooth muscle translating to functional differences in the contractile effect of adjacent smooth muscle layers. Although controversial, several investigators have reported a tonic adrenergic tone in the small bowel.[31,35,36] The tonic enteric neural regulation observed in our experiments may be caused by any of a number of neuromediators, such as cholinergic or nonadrenergic/noncholinergic mediators, or even by changes in presynaptic adrenergic receptor density or function. Further experiments are necessary to discern whether our findings represent a tonic excitatory neural input to the longitudinal smooth muscle or an inhibition of a tonic inhibitory stimulus.

Adrenergic hypersensitivity after extrinsic denervation and SBT has been observed in various experimental models. Thus, despite known interspecies and regional variability in response to denervation, we were surprised to find that we had disproved our primary hypothesis. Extrinsic denervation did not change the adrenergic response in the jejunal longitudinal smooth muscle layer, and interestingly caused a decrease in ileal longitudinal smooth muscle sensitivity to NE after enteric neural blockade with TTX. It is possible that this change in ileal longitudinal smooth muscle sensitivity to NE is mediated by changes in presynaptic myenteric adrenergic receptor number or function, or alternatively by changes at the level of the smooth muscle itself. Further experiments will be necessary to fully elucidate these pathways. These findings again highlight the rather dramatic regional differences in small bowel response to denervation.

In summary, this study showed variable contractile patterns in longitudinal smooth muscle of the canine jejunum and ileum, an adrenergic-mediated decrease in contractile activity in both the jejunum and ileum, and a decrease in basal contractile activity after blockade of enteric nerves with TTX, suggesting a role for tonic enteric neural modulation of longitudinal smooth muscle contractile activity. The adrenergic response in the jejunum was preserved after extrinsic denervation, whereas a decrease in adrenergic sensitivity in the ileum was uncovered by intrinsic neural blockade. These findings differ dramatically from those in similar experiments performed previously in canine circular jejunal and ileal smooth muscle. Adrenergic contractile mechanisms in canine longitudinal muscle of the canine jejunum and ileum are unique and differ markedly from those seen in adjacent circular muscle of the canine jejunum and ileum. These findings highlight the intraspecies regional variability of contractile function and responses to extrinsic denervation in the small bowel, and emphasize the need for a thorough study to fully understand enteric denervation and transplant physiology.

We thank Deborah Frank for her invaluable help in preparing this manuscript.

REFERENCES

1. Grant D. Intestinal transplantation: 1997 report of the international registry. Intestinal Transplant Registry. Transplantation 1999;67:1061–1064.
2. Abu-Elmagd K, Reyes J, Bond G, Mazariegos G, Wu T, Murase N, Sindhi R, Martin D, Coangelo J, Zak M, Janson D, Ezzelarab M, Dvorchik I, Parizhskaya M, Deutsch M, Demetris A, Fung J, Starzl TE. Clinical intestinal transplantation: A decade of a single center experience. Ann Surg 2001;234:404–416.
3. Sarr MG. A model of jejunoileal in vivo neural isolation of the entire jejunoileum: Transplantation and the effects on intestinal motility. J Surg Res 1989;47:266–272.
4. Johnson CP, Sarna SK, Cowles VE, Baytiyeh R, Zhu YR, Buchmann E, Bonham L, Roza AM, Adams MB. Effects of transection and reanastomosis on postprandial jejunal transit and contractile activity. Surgery 1995;117:531–537.
5. Mousa H, Bueno J, Griffiths J, Kocoshis S, Todo S, Reyes J, DiLorenzo C. Intestinal motility after small bowel transplantation. Transplant Proc 1998;30:2535–2536.
6. Pernthaler H, Nehoda H, Riedmann B, Aichberger C, Margreiter R. Altered but preserved motility of a human small bowel allograft. Transplant Proc 1996;28:2794.
7. Sugitani A, Bauer AJ, Reynolds JC, Halfter WM, Nomoto M, Starzl TE, Todo S. The effect of small bowel transplantation on the morphology and physiology of intestinal muscle. Transplantation 1997;63:186–194.
8. Quigley EM, Spanta AD, Rose SG, Lof J, Thompson JS. Long term effects of jejunoileal autotransplantation on myoelectrical activity in canine small intestine. Dig Dis Sci 1990; 35:1505–1517.
9. Nguyen BL, Thompson JS, Quigley EM. Regional variation in canine muscle mass and function. Dig Dis Sci 1995;40:1491–1497.
10. Coupar IM, Liu L. A simple method for measuring the effects of drugs on intestinal longitudinal and circular muscle. J Pharmacol Toxicol Methods 1996;36:147–154.
11. Luckensmeyer GB, Keast JR. Activation of alpha- and beta-adrenoreceptors by sympathetic nerve stimulation in the large intestine of the rat. J Physiol 1998;510:549–561.
12. Siegle ML, Buhner S, Schemann M, Schmid HR, Ehrlein HJ.

Propagation velocities and frequencies of contractions along canine small intestine. Am J Physiol 1990;258:738–744.
13. Rahman MS, Taguchi T, Nakao M, Yamada T, Suita S. Long-term results of short segmental syngeneic small intestinal transplantation: Comparison of jejunal and ileal grafts. J Pediatr Surg 1996;31:908–911.
14. Balsiger BM, Duenes JA, Ohtani N, Sarr MG. Extrinsic denervation of canine jejunum causes neuronal adrenergic and muscular cholinergic hypersensitivity. Gastroenterology 1999;116:A1075.
15. Belloli C, Re G, Arioli F, Badino P, Carcano R, Odore R, Girardi C, Beretta C. Differences between longitudinal and circular smooth muscle in β-adrenergic control of motility of isolated equine ileum. Am J Vet Res 1997;58:1422–1426.
16. Balsiger BM, Duenes JA, Ohtani N, Sarr MG. Adrenergic and cholinergic contractile mechanisms adapt after chronic extrinsic denervation of the small bowel. Gastroenterology 2000;118:A2088.
17. Shibata C, Balsiger BM, Anding WJ, Sarr MG. Adrenergic denervation hypersensitivity in ileal circular smooth muscle after small bowel transplantation in rats. Dig Dis Sci 1997; 42:2213–2221.
18. Shibata C, Murr MM, Balsiger BM, Anding WJ, Sarr MG. Contractile activity of circular smooth muscle in rats one year after small bowel transplantation: Differing adaptive response of the jejunum and ileum to denervation. J GASTROINTEST SURG 1998;2:463–472.
19. Ohtani N, Balsiger BM, Anding WJ, Duenes JA, Sarr MG. Small bowel transplantation induces adrenergic hypersensitivity in ileal longitudinal smooth muscle in rats. J GASTROINTEST SURG 2000;4:77–85.
20. Murr MM, Miller VM, Sarr MG. Contractile properties of enteric smooth msucle after small bowel transplantation in rats. Am J Surg 1996;171:212–218.
21. Nelson DK, Sarr MG, Go VLW. In vivo neural isolation of the canine jejunoileum: Temporal adaptation of enteric neuropeptides. Gut 1991;32:1336–1341.
22. Yunker AM, Galligan JJ. Extrinsic denervation increases NADPH diaphorase staining in myenteric nerves of guinea pig ileum. Neurosci Lett 1994;167:51–54.
23. Stadelmann AM, Fink JG, Telford GL, Walgenbach-Telford S, Koch TR. Nitric oxide synthase is increased following small intestinal transplantation in the rat. Neurosci Lett 1999;266:97–100.
24. Taguchi T, Guo R, Masumoto K, Nada O, Nakao M, Yanai K, Suita S. Chronological change of distribution in nitric oxide and peptidergic neurons after rat small intestinal transplantation. J Pediatr Surg 1999;34:341–345.
25. Ishii H, Kusunoki M, Yamamura T, Utsonomiya J. Effects of neuropeptides on smooth muscle contraction after small bowel transplantation. Eur Surg Res 1997;29:362–367.
26. Osinski MA, Bass P. Chronic denervation of rat jejunum results in cholinergic supersensitivity due to reduction of cholinesterase activity. J Pharmacol Exp Ther 1993;226:1684–1690.
27. Hirose R, Taguchi T, Hirata Y, Yamada T, Nada O, Suita S. Immunohistochemical demonstration of enteric nervous distribution after syngeneic small bowel transplantation in rats. Surgery 1995;117:560–569.
28. Taguchi T, Zorchta E, Sonnino RE, Guttman FM. Small intestinal transplantation in the rat: Effect on physiological properties of smooth muscle and nerves. J Pediatr Surg 1989;24:1258–1263.
29. Malmfors G, Hakanson R, Okmian L, Sundler F. Peptidergic nerves persist after jejunal autotransplantation: An experimental study in the piglet. J Pediatr Surg 1980;15:53–56.
30. Nakahata N, Nakanishi H, Suzuki T. Prejunctional inhibition mediated via alpha 2-adrenoceptors in excitatory transmission of canine small intestine. J Pharm Pharmacol 1982;34:510–513.
31. De Ponti F, Giaroni C, Cosentino M, Lecchini S, Frigo G. Adrenergic mechanisms in the control of gastrointestinal motility: From basic science to clinical applications. Pharmacol Ther 1996;69:59–78.
32. Kostka P, Sipos SN, Kwan CY, Niles LP, Daniel EE. Identification and characterization of presynaptic and postsynaptic beta adrenoreceptors in the longitudinal smooth muscle/myenteric plexus of dog ileum. J Pharmacol Exp Ther 1989;251:305–310.
33. Lu G, Sarr MG, Szurszewski JH. Effect of extrinsic denervation in a canine model of jejunoileal autotransplantation on mechanical and electrical activity of jejunal circular smooth muscle. Dig Dis Sci 1997;42:40–46.
34. Sarna SK. Gastrointestinal longitudinal muscle contractions. Am J Physiol 1993;265:156–164.
35. Tonini M, Lecchini S, Frigo G, Crema A. Action of tetrodotoxin on spontaneous electrical activity of some smooth muscle preparations. Eur J Pharmacol 1974;29:236–240.
36. McIntyre AS, Thompson DG, Day S, Burnham WR, Walker ER. Modulation of human upper intestinal nutrient transit by a beta adrenoreceptor mediated pathway. Gut 1992;33:1062–1070.

Diagnostic Laparoscopy and Laparoscopic Ultrasound for Staging of Patients With Malignant Proximal Bile Duct Obstruction

Esther H.B.M. Tilleman, M.D., Steve M.M. de Castro, Olivier R.C. Busch, M.D. Willem A. Bemelman, M.D., Thomas M. van Gulik, M.D., Huug Obertop, M.D., Dirk J. Gouma, M.D.

Tumor staging in patients with a malignant obstruction of the proximal bile duct is focused on selecting patients who could benefit from a resection. Diagnostic laparoscopy, which has proved its value in several gastrointestinal malignancies, has been used routinely at our hospital since 1993 in patients with a malignant obstruction of the proximal bile duct, although data in the literature with regard to its additional value are conflicting. Therefore the diagnostic accuracy of diagnostic laparoscopy in patients with malignant proximal bile duct obstruction was evaluated. From January 1993 to May 2000, diagnostic laparoscopy was performed in 110 patients (61 males and 49 females), with a mean age of 60 years (range 30 to 80 years), who had a suspected malignant proximal bile duct tumor and in whom "potential resectability" was demonstrated by means of conventional radiologic staging methods (i.e., ultrasound combined with Doppler imaging, CT, endoscopic retrograde cholangiopancreatography, and percutaneous transhepatic cholangiography). Laparoscopy revealed histologically proved incurable disease in 44 (41%) of the 110 patients (31 with metastases and 13 with extensive tumor ingrowth). Laparoscopic ultrasound imaging, however, revealed histologically proved incurable disease in one patient (1%), thereby preventing exploratory laparotomy in 46 because these patients had already been treated by palliative endoscopic stent placement. The remaining 65 patients were staged as having a resectable tumor and underwent surgical exploration. Thirty patients had an unresectable tumor (distant metastases in five; tumor ingrowth in surrounding tissues in 24) or benign disease (one patient). Sensitivity and negative predictive value of diagnostic laparoscopy for detecting unresectable disease were 60% and 52%, respectively. Diagnostic laparoscopy avoided unnecessary laparotomy in 41% of patients with a malignant proximal bile duct obstruction considered resectable according to conventional imaging studies. The additional value of laparoscopic ultrasound was limited. Therefore diagnostic laparoscopy should be performed routinely in the workup of patients with a potentially resectable proximal bile duct tumor. (J GASTROINTEST SURG 2002;6:426–431.)

KEY WORDS: Bile duct neoplasms, laparoscopy; neoplasm staging/methods

A "malignant" proximal bile duct obstruction can be caused by a proximal bile duct carcinoma or a gallbladder carcinoma infiltrating the liver hilus. In general, a gallbladder carcinoma is easily recognized. However, in a small subgroup of patients, differentiation between a cholangiocarcinoma and a gallbladder carcinoma with infiltration into the proximal bile ducts, metastasis from another tumor, or even a benign lesion is not always possible. Despite the evolution of radiologic imaging techniques, such as ultrasound combined with Doppler imaging, endoscopic retrograde cholangiopancreatography (ERCP), enhanced CT, and magnetic resonance imaging, in the selection of patients suitable for tumor resection, all imaging modalities have their limitations in detecting small superficial liver metastases and peritoneal tumor deposits or tumor ingrowth into the hepatoduodenal ligament and/or adjacent vascular structures. Therefore the surgeon dealing with malignant proximal bile duct obstruction is often faced with a significant discrepancy between the findings on preoperative investigations and those apparent at the

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (oral presentation).

From the Department of Surgery (E.H.B.M.T., S.M.M.D.C., O.R.C.B., W.A.B., T.M.V.G., H.O., D.J.G.), Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

Reprint requests: D.J. Gouma, Academic Medical Center, Department of Surgery, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. e-mail: d.j.gouma@amc.uva.nl

Published by Elsevier Science Inc.

1091-255X/02/$—see front matter
PII: S1091-255X(02)00005-7

time of laparotomy.[1,2] Improvement in preoperative staging is mandatory to improve the assessment of tumor stage and to avoid an unnecessary laparotomy when palliation can be achieved by means of endoscopic or percutaneous placement of a biliary endoprosthesis.[3,4] During the past decade, nonsurgical palliation has been the procedure of choice at our institution, whereas others favor a surgical approach.[5,6]

The additional value of diagnostic laparoscopy in the preoperative staging of gastrointestinal malignancies has previously been reported.[7–11] Small superficial liver metastases and peritoneal tumor deposits or local ingrowth can be detected and biopsies done under direct vision.[7,12,13] Laparoscopic ultrasound allows inspection of local tumor invasion in patients with vascular and regional nodal involvement and distant metastatic spread to the liver. However, histologic confirmation of these findings can be difficult to obtain.[14,15]

Information on the additional value of diagnostic laparoscopy for malignant proximal bile duct obstruction is limited. In a pilot study at our institution, incurable disease was diagnosed in 40% of patients by means of diagnostic laparoscopy.[16] Because data in the literature are conflicting, we decided to evaluate the usefulness of diagnostic laparoscopy and laparoscopic ultrasound imaging in predicting resectability in 110 consecutive patients with a malignant proximal bile duct obstruction who were judged to have resectable disease after standard radiologic staging. The accuracy of laparoscopic staging in detecting unresectable disease and preventing unnecessary surgical exploration was studied.

PATIENTS AND METHODS

Between January 1993 and May 2000, diagnostic laparoscopy was performed in 110 consecutive patients at the Academic Medical Center, Amsterdam, The Netherlands, who were found to have obstructive jaundice due to a proximal bile duct obstruction after radiologic staging demonstrated a suspected bile duct malignancy without distant metastases. The diagnostic workup consisted of a transabdominal ultrasound examination combined with Doppler imaging. ERCP was performed in most patients with obstructive jaundice, and at least one endoprosthesis was placed if possible. Before 1995 conventional CT scanning was performed, and after 1995 spiral CT scanning was used. Magnetic resonance cholangiopancreatography and endoscopic ultrasound were only performed in a select group of patients in whom controversy remained regarding the intrahepatic segmental tumor growth after radiologic staging, because the value of these investigations in predicting the resectability of these tumors has not been proved. Patients were classified according to the Bismuth-Corlette classification into types I to IV.[17]

Diagnostic laparoscopy was performed under general anesthesia as previously described.[15,18] In short, a CO_2 pneumoperitoneum was induced and, with the use of three 10 to 11 mm trocars (umbilical, and left and right subcostal), the abdominal cavity was explored to determine the type of tumor (i.e., cancer of the gallbladder or cholangiocarcinoma) and assess each patient for the presence of peritoneal or hepatic deposits and for extensive malignant infiltration of the hepatoduodenal ligament. Dissection of the hepatoduodenal ligament and elevation of the hilar plate were not performed. If no histologically proved distant metastases or tumor ingrowth into the hepatoduodenal ligament was found, laparoscopic ultrasound imaging was performed. A 7.5 MHz linear-array ultrasound probe (UST5522-7.5; Aloka Co., Tokyo, Japan) was used to examine the liver for intrahepatic metastases, to evaluate the portal vein, and to investigate the celiac axis for lymph node metastases. Biopsy samples of suspected metastatic lesions were obtained under direct laparoscopic vision or by laparoscopic ultrasound guidance using biopsy forceps or True-cut (Travenol-Baxter Healthcare Corp., Deerfield, Illinois) and Rostex (Ursus Konsult AB, Stockholm, Sweden) biopsy needles. All tumors were restaged after laparoscopy. A tumor was considered unresectable if metastatic disease or extrahepatic tumor ingrowth, detected during laparoscopy, was proved histologically. In all patients with a tumor judged to be resectable or in patients in whom metastatic disease, locoregional tumor ingrowth, or local extensive gallbladder tumor shown at laparoscopy could not be proved histologically, an exploratory laparotomy was performed, preferably within 4 weeks after laparoscopy. Preoperatively, external irradiation therapy (3×3.5 Gy) was administered to devitalize detached tumor cells in the bile, to prevent implantation of seeding metastases as described previously.[19,20]

Patients with an unresectable tumor at laparoscopy or laparotomy underwent nonsurgical palliation that included placement of a biliary endoprosthesis or Wallstent (Schneider Europe AG, Bulack, Switzerland). In two of these patients with a high risk of gastric outlet obstruction that was missed on conventional CT scan (before 1997) but discovered at laparoscopy, gastroenterostomy was performed. When curative resection was possible, a hilar resection was performed with or without (extended) hemihepatectomy and almost always included resection of the caudate lobe.

RESULTS

Diagnostic laparoscopy was performed in 110 patients (61 men and 49 women, median age 60 years [range 30 to 80 years]). Laparoscopic ultrasound imaging was performed in 74 patients. In the other 36 patients, the procedure was judged to be superfluous because of evidence of unresectability as diagnosed by laparoscopy alone.

Diagnostic laparoscopy alone demonstrated histologically proved unresectable disease in 44 patients (40%): metastasis in 31 patients, locoregional tumor ingrowth in 10 patients, and unresectable gallbladder carcinoma in three patients (Table 1). Metastases were localized at the surface of the liver in 10 patients, on the peritoneum in 10, on the diaphragm in eight, and in the omentum in three. In three patients, laparoscopic inspection was incomplete because of massive adhesions; these patients were sent for surgical exploration.

Laparoscopic ultrasound imaging was performed in 74 patients, 12 of whom already had histologically proved metastases detected on laparoscopy alone. Among the other 62 patients, metastasis was suspected in 11 patients and locally extensive disease in eight, but histologic proof could only be obtained in one patient. Mean and median operating time for diagnostic laparoscopy was 60 minutes and ranged from 15 to 145 minutes; approximately half of the time was used for the laparoscopic ultrasound imaging.

Morbidity was low (3%); postoperative bleeding requiring repeat laparotomy occurred in two patients (2%) and one patient (1%) had an allergic reaction to iodine. No deaths occurred. The median hospital stay for the laparoscopic procedure was 2 days with a range of 1 to 11 days.

Patients with tumors considered potentially resectable after diagnostic laparoscopy and laparoscopic ultrasound imaging (n = 65) underwent surgical exploration. Unfortunately, in 31 of these patients (46%) the tumor was not resected because of metastases (n = 5), locoregional tumor ingrowth (n = 21), or locally unresectable gallbladder carcinoma (n = 3), and in one patient the resection was not completed because of benign disease (sclerosing cholangitis). Distant metastases were found in the liver in three patients and in distant lymph nodes in two (Table 2). In Fig. 1 a flow chart is presented outlining the results of laparoscopic staging of 110 patients with a proximal bile duct obstruction. Eighteen patients with suspicion of (but not histologically proved) unresectable disease detected at laparoscopic ultrasound imaging underwent surgical exploration, and in seven patients (39%) a resection was performed, that was in 61% microscopically radical.

Of the 30 patients with unresectable disease at laparotomy, a gastroenterostomy was performed in two patients and a hepaticojejunostomy in combination with a gastroenterostomy in one. Of the 35 patients who underwent a resection, in 15 patients a local hilar resection was performed and in 20 patients a hilar resection with hemihepatectomy was used. In 22 patients (59%) the resection was microscopically radical.

The median interval between laparoscopy and laparotomy was 36 days (range 17 to 84 days). The sensitivity and negative predictive value of diagnostic laparoscopy for the detection of unresectable disease were 60% and 52%, respectively.

DISCUSSION

In this series of 110 consecutive patients, diagnostic laparoscopy increased the yield of radiologic assessment of distant metastases and locoregional ingrowth in 41% of the patients. As a result, an unnecessary exploratory laparotomy could be avoided in these patients because almost all of them were palliated by nonsurgical means.

Laparoscopic ultrasound imaging was of limited additional value in this study. The suspicion of unresectability mainly because of local ingrowth could only be proved by histologic examination in 1 of 19 patients. The fact that 7 of 18 remaining patients

Table 1. Laparoscopic identification and histological proof of unresectable disease

	Laparoscopy (*n* = 110)
Metastases	
Liver	10
Peritoneum	10
Diaphragm	8
Omentum	3
Loco regional tumor ingrowth	13
Total (avoided laparotomy)	44 (40%)

Table 2. Perioperative identification of unresectable disease

Metastases	(*n* = 65)
Liver	3
Lymph nodes	2
Local extensive gallbladder tumor	3
Loco regional tumor ingrowth	21
Benign disease	1
Total unresected	30 (46%)

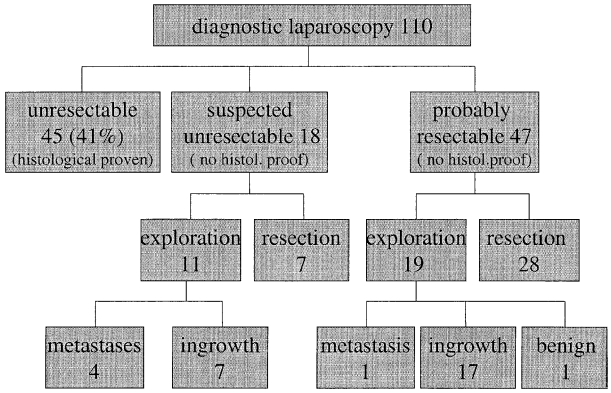


Fig. 1. Laparoscopic staging of 110 patients with a proximal bile duct obstruction.

could undergo a resection underlines the need for histologic proof in cases of metastases or local unresectable disease. Furthermore, the inability to obtain histologic proof of (vascular) ingrowth potentially accounts for the limited additional value of laparoscopic ultrasound.[21,22]

Diagnostic laparoscopy is only useful in patients who could benefit from nonsurgical palliation because in these patients it makes sense to avoid an exploratory laparotomy. At our institution surgical palliation is performed only in select patients and there were three patients in the present series. Others favor nonsurgical palliation, although this is associated with considerable procedure-related morbidity and mortality and late complications such as obstruction or cholangitis.[5] There is currently insufficient evidence to support the use of one palliative procedure over the other, and the choice seems to be dependent on the experience within a particular center.

Some studies show that endoscopic endoprosthesis placement is associated with a high rate of failure compared to percutaneous placement.[23] On the other hand, percutaneus endoprosthesis placement can be associated with more complications, and therefore the endoscopic route is our first choice for nonsurgical biliary drainage.

In forty-six percent of the patients in the present series, diagnostic laparoscopy had not detected the metastasis or extensive tumor ingrowth that was found during exploratory laparotomy. Patients had unresectable disease mainly due to lymph node metastases and vascular involvement or extensive biliary involvement, as has been reported by others.[6,24] More extensive dissection at laparoscopy could have detected one or two of these findings, probably at the cost of a longer procedure and greater overall risk. However, some improvement in the yield of laparoscopy can be obtained, as 5% of the unresectable tumors were due to metastases that could have possibly been detected at laparoscopy.

In recent years, preoperative radiologic staging has improved substantially with the introduction of the spiral CT with 3 mm slides, and endoscopic ultrasonography has also shown improvement. These diagnostic improvements could have led to a higher detection rate for metastases that were missed in our series. Consequently a reduction in the additional value of diagnostic laparoscopy in staging proximal bile duct malignancies can be expected in the future, similar to what has been shown for distal bile duct obstructions caused by periampullary tumors. Differentiation between gallbladder and proximal bile duct carcinoma on radiologic assessment is sometimes difficult. In the present study, before laparoscopy, there was doubt about the localization of the tumor in at least 20% of patients.

Because biopsies performed during diagnostic laparoscopy often show false negative benign disease and increase the chances of seeding metastases, in the present series no biopsy specimens were taken from tumors presumed to be resectable. Resection was performed in 13 patients who turned out to have benign disease after histopathologic examination of the resection specimen. In a previous study of 132 resections for presumed proximal bile duct tumor, a 15% false positive rate of malignancy was reported in patients with a presumed malignant proximal bile duct obstruction.[25]

At our institution, diagnostic laparoscopy and laparotomy in general are not performed in one session for logistic reasons. One additional consequence of not performing diagnostic laparoscopy and laparotomy in one session was that the total hospital stay for those patients who underwent a laparotomy was longer, because they were eventually admitted a second time for the resection. Therefore the potential financial benefit was not as great as would be possible if diagnostic laparoscopy and laparotomy were performed in one session.

In summary, diagnostic laparoscopy without laparoscopic ultrasound is useful in staging patients with proximal bile duct tumors because in a considerable number of patients an unnecessary laparotomy can be avoided since nonsurgical palliation is available.

REFERENCES

1. Helling TS. Carcinoma of the proximal bile duct. J Am Coll Surg 1994;178:97–106.
2. Kremer B, Henne-Bruns D, Vogel I, et al. Impact of staging on treatment of biliary carcinoma. Endoscopy 1993;25:92–99.
3. Cuesta MA, Meijer S, Borgstein PJ. Laparoscopy and assessment of digestive tract cancer. Br J Surg 1992;79:486–487.

4. Garner PD, Hall LD, Johnstone PA. Palliation of unresectable hilar cholangiocarcinoma. J Surg Oncol 2000;75:95–97.
5. Jarnagin WR, Burke E, Powers C, et al. Intrahepatic biliary enteric bypass provides effective palliation in selected patients with malignant obstruction at the hepatic duct confluence. Am J Surg 1998;175:453–460.
6. England RE, Martin DF. Endoscopic and percutaneous intervention in malignant obstructive jaundice. Cardiovasc Intervent Radiol 1996;19:381–387.
7. Cuesta MA, Meijer S, Borgstein PJ, et al. Laparoscopic ultrasonography for hepatobiliary and pancreatic malignancy. Br J Surg 1993;80:1571–1574.
8. John TG, Wright A, Allan PL, et al. Laparoscopy with laparoscopic ultrasonography in the TNM staging of pancreatic carcinoma. World J Surg 1999;23:870–881.
9. Callery MP, Strasberg SM, Doherty GM, et al. Staging laparoscopy with laparoscopic ultrasonography: Optimizing resectability in hepatobiliary and pancreatic malignancy. J Am Coll Surg 1997;185:33–39.
10. Pietrabissa A, Caramella D, Di Candio G, et al. Laparoscopy and laparoscopic ultrasonography for staging pancreatic cancer: Critical appraisal. World J Surg 1999;23:998–1002.
11. Pratt BL, Greene FL. Role of laparoscopy in the staging of malignant disease. Surg Clin North Am 2000;80:1111–1126.
12. John TG, Greig JD, Crosbie JL, et al. Superior staging of liver tumors with laparoscopy and laparoscopic ultrasound [see comments]. Ann Surg 1994;220:711–719.
13. Anderson DN, Campbell S, Park KG. Accuracy of laparoscopic ultrasonography in the staging of upper gastrointestinal malignancy. Br J Surg 1996;83:1424–1428.
14. Gouma DJ, de Wit LT, Nieveen VD, et al. Laparoscopic ultrasonography for staging of gastrointestinal malignancy [Review]. Scand J Gastroenterol 1996;218(Suppl):43–49.
15. van Delden OM, Smits NJ, Bemelman WA, et al. Comparison of laparoscopic and transabdominal ultrasonography in staging of cancer of the pancreatic head region. J Ultrasound Med 1996;15:207–212.
16. Van Dijkum EJ, de Wit LT, van Delden OM, et al. Staging laparoscopy and laparoscopic ultrasonography in more than 400 patients with upper gastrointestinal carcinoma. J Am Coll Surg 1999;189:459–465.
17. Bismuth H, Corlette MB. Intrahepatic cholangioenteric anastomosis in carcinoma of the hilus of the liver. Surg Gynecol Obstet 1975;140:170–178.
18. Bemelman WA, de Wit LT, van Delden OM, et al. Diagnostic laparoscopy combined with laparoscopic ultrasonography in staging of cancer of the pancreatic head region. Br J Surg 1995;82:820–824.
19. Gerhards MF, Gonzalez DG, ten Hoopen-Neumann H, et al. Prevention of implantation metastases after resection of proximal bile duct tumours with pre-operative low dose radiation therapy. Eur J Surg Oncol 2000;26:480–485.
20. ten Hoopen-Neumann H, Gerhards MF, van Gulik TM, et al. Occurrence of implantation metastases after resection of Klatskin tumors. Dig Surg 1999;16:209–213.
21. Gouma DJ, Nieveen V, Dijkum EJ, de Wit LT, Obertop H. Laparoscopic staging of biliopancreatic malignancy [Review]. Ann Oncol 1999; 10(Suppl 4):33–36.
22. van Delden OM, de Wit LT, Nieveen V, et al. Value of laparoscopic ultrasonography in staging of proximal bile duct tumors. J Ultrasound Med 1997;16:7–12.
23. Cheung KL, Lai EC. Endoscopic stenting for malignant biliary obstruction. Arch Surg 1995;130:204–207.
24. Nordback IH, Pitt HA, Coleman J, et al. Unresectable hilar cholangiocarcinoma: Percutaneous versus operative palliation. Surgery 1994;115:597–603.
25. Gerhards MF, Vos P, van Gulik TM, et al. Incidence of benign lesions in patients resected for suspicious hilar obstruction. Br J Surg 2001;88:48–51.

Invited Discussion—Expert Commentator

Henry A. Pitt, M.D. (Milwaukee, WI): The paper by Tilleman and colleagues concluded that staging laparoscopy with ultrasonography should be performed routinely in patients with perihilar cholangiocarcinoma. They report a high specificity but a low sensitivity; however, they do not report accuracy, which must have been quite modest. A major reason for their positive conclusion was the finding of liver or peritoneal metastases at laparaoscopy in 36% of their patients, and this percentage was greater than 40% overall when five additional patients were discovered a laparatomy to have metastatic disease. This percentage of metastatic disease in perihilar cholangiocarcinoma is much higher than in many series.

One explanation for this observation is that the authors included some patients with gallbladder cancer in this series. Patients with gallbladder cancer presenting with bile duct obstruction do have a high likelihood of metastatic disease and a low resectability rate, and therefore that subset of patients should undergo routine laparascopy. And if you are smart enough to make that diagnosis on a noninvasive test, that would be a good reason to go ahead with laparascopy. However, in most series of perihilar cholangiocarcinoma, the incidence of metastatic disease is low, but a significant percentage, perhaps 15 or 20% of these tumors, can be deemed unresectable on the basis of extensive bile duct involvement, for example, the Bismuth type IV,

and/or definite vascular encasement on preoperative imaging studies.

Therefore, I would suggest that in perihilar cholanggiocarcinoma, staging laparascopy should be employed selectively, perhaps in patients with Bismuth type III tumors and in those in whom there is a suspicion of main portal vein or contralateral portal vein branch or hepatic artery encasement.

Mechanisms of Impaired Gallbladder Contractile Response in Chronic Acalculous Cholecystitis

Anders R. Merg, M.D., Scott E. Kalinowski, B.S., Marilyn M. Hinkhouse, B.S., Frank A. Mitros, M.D., Kimberly S. Ephgrave, M.D., Joseph J. Cullen, M.D.

The mechanisms involved in the impaired gallbladder contractile response in chronic acalculous cholecystitis are unknown. To determine the mechanisms that may lead to impaired gallbladder emptying in chronic acalculous cholecystitis, gallbladder specimens removed during hepatic resection (controls) and after cholecystectomy for chronic acalculous cholecystitis were attached to force transducers and placed in tissue baths with oxygenated Krebs solution. Electrical field stimulation (EFS) (1 to 10 Hz, 0.1 msec, 70 V) or the contractile agonists, CCK-8 (10^{-9} to 10^{-5}) or K^+ (80 mmol/L), were placed separately in the tissue baths and changes in tension were determined. Patients with chronic acalculous cholecystitis had a mean gallbladder ejection fraction of 12% ± 4%. Pathologic examination of all gallbladders removed for chronic acalculous cholecystitis revealed chronic cholecystitis. Spontaneous contractile activity was present in gallbladder strips in 83% of control specimens but only 29% of gallbladder strips from patients with chronic acalculous cholecystitis ($P < 0.05$ vs. controls). CCK-8 contractions were decreased by 54% and EFS-stimulated contractions were decreased by 50% in the presence of chronic acalculous cholecystitis ($P < 0.05$ vs. controls). K^+-induced contractions were similar between control and chronic acalculous cholecystitis gallbladder strips. The impaired gallbladder emptying in chronic acalculous cholecystitis appears to be due to diminished spontaneous contractile activity and decreased contractile responsiveness to both CCK and EFS. (J GASTROINTEST SURG 2002;6:432–437.)

KEY WORDS: Acalculous cholecystitis, motility, gallbladder

A small subset of patients having classic symptoms of biliary colic do not have detectable gallstones at the time of ultrasonography. In these patients, cholecystokinin (CCK) cholescintigraphy confirms the presence of a patent cystic duct and permits functional evaluation of gallbladder emptying in response to the hormonal stimulus. Decreased gallbladder emptying, known as biliary dyskinesia or chronic acalculous cholecystitis, is a syndrome in which gallbladder hypomotility produces symptoms suggestive of biliary colic in the absence of gallstones.[1] Decreased gallbladder emptying is usually diagnosed by a CCK-stimulated ejection fraction of less than 35% or scintigraphic or nonemptying of the gallbladder. According to these definitions, chronic acalculous cholecystitis represents approximately 5% of patients with gallbladder disease.[2] In a review by Canfield et al.,[3] the combined results of 14 studies since 1975 demonstrate that among symptomatic patients with an ejection fraction of less than 50%, as determined by CCK scintigraphy, 97% show improvement or become asymptomatic after cholecystectomy.

The pathogenesis of this disease is unclear. Recent studies suggest that the gallbladder smooth muscle impairment in chronic acalculous cholecystitis may be caused by a distinct smooth muscle defect that appears to be different from the smooth muscle impairment in gallbladders with cholesterol stones. Amaral et al.[4] demonstrated that gallbladder contractions induced by CCK-8 were decreased in gallbladders with chronic acalculous cholecystitis compared to those with pigment stones. In vitro smooth muscle cell contraction was also impaired in chronic acalculous cholecystitis after stimulation with G-protein activators and with

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20-23, 2001 (poster presentation).

From the Departments of Pathology (F.A.M.) and Surgery (A.R.M., S.E.K., K.S.E., J.J.C.), University of Iowa College of Medicine, and the Veterans Administration Medical Center (M.M.H., K.S.E., J.J.C.), Iowa City, Iowa.

Supported by the Veterans Administration Research Service and National Institutes of Health training grant HLO 7485-19.

Reprint requests: Joseph J. Cullen, M.D., 4622 JCP, University of Iowa Hospitals and Clinics, Iowa City, IA 52242.

Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(01)00061-0

a protein kinase C activator; however, the CCK receptors in muscle membranes of gallbladders with chronic acalculous cholecystitis were normal. From this investigation it was concluded that impaired smooth muscle contractility in chronic acalculous cholecystitis appears to reside in the contractile apparatus and is not due to CCK binding capacity. Gallbladder emptying, however, depends on both smooth muscle contractile function and neural efferent motor pathways regulating gallbladder contractions.[5]

The aim of our study was to determine the mechanisms that might lead to the impaired gallbladder emptying in chronic acalculous cholecystitis. We hypothesized that the impaired gallbladder emptying in chronic acalculous cholecystitis was due to impaired smooth muscle contractility to contractile agonists and nerve-induced contractile responses.

PATIENTS AND METHODS

Patients

Seven patients (3 men and 4 women, mean age 42 ± 7 years) with recurrent episodes of epigastric or right upper quadrant pain typical of biliary colic underwent laparoscopic cholecystectomy for the diagnosis of chronic acalculous cholecystitis. The diagnosis of chronic acalculous cholecystitis was made after all patients were studied with ultrasonography and CCK cholescintigraphy. In all patients, gallbladder ultrasonography failed to demonstrate cholithiasis, and cholescintigraphy revealed decreased gallbladder ejection fractions of less than 30%. Patients with a history of acute acalculous cholecystitis were excluded. Scintigraphy was performed using a ^{99m}Tc mebrofenin (iminodiacetic acid derivative) imaging agent, and Sincalide to reproduce the CCK biological activities. Briefly, patients were kept fasting for 4 hours before the hepatobiliary scintigraphy was initiated. A total 5 mCi of ^{99m}Tc mebrofenin was injected intravenously after which 60 images/sec of the right upper quadrant region were acquired. Serial images every 5 minutes for the first hour were obtained with a Siemens-Diacam gamma camera (Hoffman Estates, Illinois). The gallbladder ejection fraction was then measured by administering Sincalide 1 hour after ^{99m}Tc mebrofenin injection. Gamma rays from the gallbladder region were counted before the Sincalide injection and 30 minutes after the injection. The ejection fraction was calculated as the pre-Sincalide injection count minus the 30-minutes post-Sincalide injection gamma count divided by the pre-Sincalide injection gamma count.

Gallbladder specimens were also obtained from six asymptomatic patients (3 men and 3 women, mean age 49 ± 10 years) with normal findings on ultrasound examination of the gallbladder, who were undergoing hepatic resection for metastatic malignancies served as control specimens. In both control subjects and patients with chronic acalculous cholecystitis, the gallbladder was removed within 15 minutes after the cystic artery was ligated. In both the control and chronic acalculous cholecystitis groups, the bile from the gallbladders was analyzed and found to contain no gallstones, cholesterol crystals, or cholesterolosis. The gallbladder specimens were then immediately placed in ice-cold oxygenated Krebs solution.

Conduct of Tests

The gallbladder specimens were removed and washed with Krebs solution (consisting of [in mmol/L] 138.5 sodium, 4.6 potassium, 2.5 calcium, 1.2 magnesium, 125 chloride, 21.9 bicarbonate, 1.2 phosphate, 1.2 sulfate, and 11.5 glucose), and maintained at 37° C and gassed with 95% O_2/5% CO_2. The gallbladder was opened as a flat sheet, and the serosa and mucosa were removed under a dissecting microscope and strips approximately 2 mm wide were cut in a transverse orientation. These strips were mounted on rack-and-pinion clamps to permit stretching and to record smooth muscle contractions. The strips were placed in 5 ml organ baths containing Krebs solution at 37° C continuously bubbled with 95% O_2/5% CO_2 and allowed to equilibrate. During equilibration of the strips, the development of spontaneous contractile activity was noted to be either present or absent. Electrical field stimulation (EFS) and dose-response studies to contractile agonists were performed on separate gallbladder strips. For the EFS experiments, strips were stretched to an initial tension of 3 g and equilibrated for 1 to 2 hours with occasional washings as described by Chen et al.[6] Gallbladder strips develop steady active tension during the equilibration period. Basal active tension and changes in tension were obtained after subtracting passive tension from all measurements. Passive tension was obtained at the end of the experiment after administration of 20 mmol/L ethylenediaminetetra-acetic acid (Sigma Chemical, St. Louis, Missouri). EFS was delivered from a Grass S48 (Grass Instrument Co., Quincy, Massachusetts) stimulator and neurally mediated relaxation of gallbladder muscle strips were measured in response to increasing frequencies (1-10 Hz) of EFS (70 V, 1-msecond pulse duration for 20 sec). Recently it has been demonstrated that a short-train stimulus applied to gallbladder smooth muscle strips in vitro evokes a contraction from the end of the stimulus.[7] This nonadrenergic noncholinergic nerve-induced response coincides with depolarization of the smooth muscle.

For the dose-response studies the optimal length (L_o) of each muscle strip was determined by estab-

lishing the initial length (L_i) and then progressively stretching the muscle strip in 0.5 mm increments. At each increment of stretch, the muscle strip was allowed to stabilize and then challenged with 10^{-4} mol/L bethanechol, followed by 3 washes, and 20 minutes of equilibration, until the active contractile response was achieved. All subsequent experiments were performed with the muscle strips equilibrated at their respective L_o, the length at which active force was maximum. Cumulative dose-response studies were then performed for cholecystokinin octapeptide (CCK-8) 10^{-12} to 10^{-5} mol/L (Sigma Chemical). Additionally, in other gallbladder strips, the contractile responses to maximally effective concentrations of potassium (80 mmol/L) were also tested. At the end of each study, the length of each strip was measured, blotted lightly, and weighed. The cross-sectional area of each strip was calculated from length and weight data by assuming that the density of smooth muscle is 1.05 g/cm^3.

Analysis of Data

Analysis of variance (ANOVA) with Tukey's test was used to determine differences between the control and chronic acalculous cholecystitis groups. Chi-square analysis was used to determine differences between the control and chronic acalculous cholecystitis groups in terms of spontaneous contractile activity. The protocol for use of human gallbladder tissue was approved by the University of Iowa Institutional Review Board for Human Subjects on February 13, 1999.

RESULTS

In the patients with chronic acalculous cholecystitis, cholescintigraphy demonstrated a mean gallbladder ejection fraction of 12% ± 4%. Pathologic examination of gallbladders removed during hepatic resection revealed no evidence of inflammation or cholecystitis (Fig. 1, *A*). Pathologic examination of all gallbladder specimens for chronic acalculous cholecystitis revealed inflammatory cell infiltrates and Rokitansky-Aschoff sinuses consistent with chronic cholecystitis (Fig. 1, *B*). All of the patients with chronic acalculous cholecystitis had resolution of their presenting symptoms after laparoscopic cholecystectomy.

Chronic acalculous cholecystitis was associated with a decreased incidence of spontaneous contractile activity in the gallbladder specimens (Fig. 2). Among the control gallbladders, five (83%) of six gallbladder specimens developed spontaneous contractile activity when the muscle strips were attached to force transducers in the oxygenated tissue bath. In contrast, only two (29%) of seven gallbladder specimens developed spontaneous contractile activity during the in vitro studies ($P < 0.05$ vs. control).

Gallbladder smooth muscle strips from chronic acalculous cholecystitis specimens had decreased contractile responses to CCK-8 (10^{-12} to 10^{-5} mol/L) (Fig. 3). The maximal contraction of control gallbladder strips in response to CCK-8 (10^{-5} mol/L) was 37.1 ± 9.3 g/cm^2. The maximal contraction response to CCK-8 (10^{-5} mol/L) was decreased to 18.1 ± 3.6 g/cm^2 in gallbladder strips from patients with chronic acalculous cholecystitis ($P < 0.05$ vs. control).

Gallbladder smooth muscle strips from chronic acalculous cholecystitis specimens also had a decreased contractile response to EFS (1 to 10 Hz) (Fig. 4). The maximal contraction of control gallbladder strips to EFS (10 Hz) was 28.4 ± 6.5 g/cm^2. The maximal contraction response to EFS (10 Hz) was decreased to 14.2 ± 3.3 g/cm^2 in gallbladder strips from patients with chronic acalculous cholecystitis ($P < 0.05$ vs. control).

Gallbladder smooth muscle strips from chronic acalculous cholecystitis specimens had a slight decreased contractile response to K^+, which did not achieve statistical significance. Gallbladder strips contracted to 45.1 ± 8.0 g/cm^2 in control gallbladders in response to K^+ (80 mmol/L), which was slightly decreased but similar to the response in strips from chronic acalculous cholecystitis specimens, which contracted to 28.6 ± 5.8 g/cm^2 ($P > 0.05$ vs. control).

DISCUSSION

Our study demonstrates that gallbladders removed for chronic acalculous cholecystitis are associated with inflammation consistent with chronic cholecystitis. Additionally, in vitro studies demonstrate that gallbladders from patients with chronic acalculous cholecystitis have an increased absence of spontaneous contractile activity, a decreased response to the contractile agonist CCK, and a decreased contractile response to EFS, when compared to gallbladders without disease.

Our study compliments the recent study by Amaral et al.,[4] which also demonstrated a decreased contractile response in gallbladders from patients with chronic acalculous cholecystitis. In their study comparing gallbladders with chronic acalculous cholecystitis with those removed for cholesterol or pigment stones, contraction was also impaired after stimulation with G-protein activators and with a protein kinase C activator. However, the CCK receptors in muscle membranes of gallbladders with chronic acalculous cholecys-

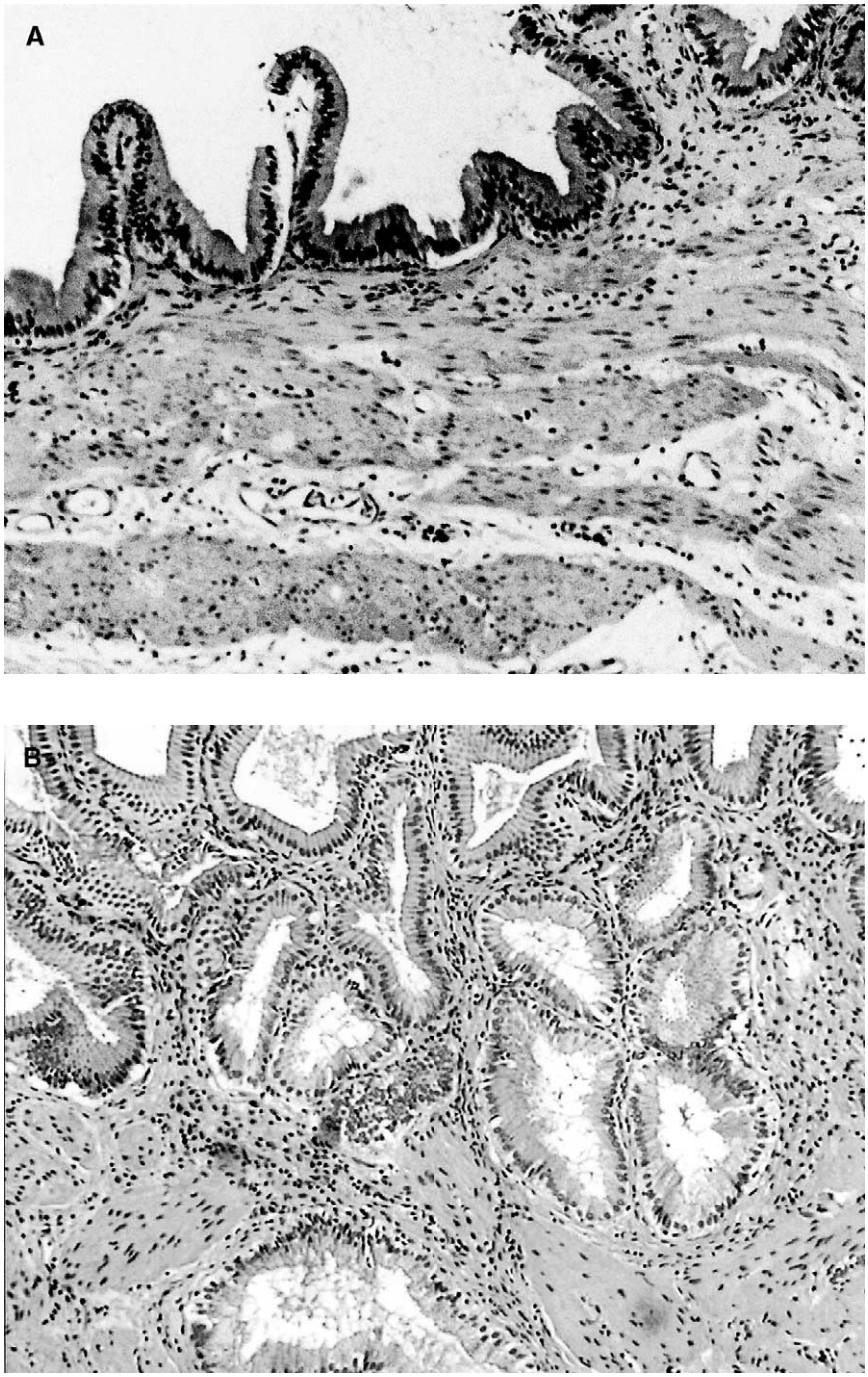


Fig. 1. A, Histologic section of a control gallbladder removed during hepatic resection. Note normal wall thickness and absence of inflammation or evidence of cholecystitis. **B**, Histologic section of a gallbladder removed for chronic acalculous cholecystitis. Note thickened muscularis with scattered lymphocytic infiltrate. Additionally, Rokitansky-Aschoff sinuses are identified, which is consistent with the diagnosis of chronic cholecystitis.

titis were normal. Our present study differs from this previous study in that we used scintigraphy instead of sonography to confirm the diagnosis of chronic acalculous cholecystitis, and we compared chronic acalculous cholecystitis gallbladder specimens with gallbladders removed during hepatic resection that contained no cholesterol or pigment stones. Although we did not evaluate CCK receptors in the gallbladder specimens we used for this study, our data, combined with data from the study by Amaral et al.,[4] implicate impaired

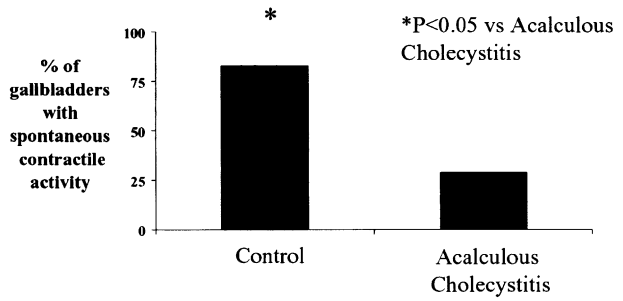


Fig. 2. Percentage of gallbladder strips with spontaneous contractile activity in control and chronic acalculous cholecystitis gallbladder specimens. Control gallbladders demonstrated an increased percentage of smooth muscle strips with spontaneous contractile activity. *$P < 0.05$ vs. control; chi-square analysis.

smooth muscle contractility in chronic acalculous cholecystitis, which appears to reside in the contractile apparatus and is not due to CCK binding capacity.

Gallbladder emptying depends on both smooth muscle function and neural efferent motor pathways regulating gallbladder contractile activity. The intrinsic cholinergic innervation of the gallbladder is important for gallbladder contractility and subsequent emptying. Atropine decreases meal-stimulated gallbladder emptying,[8] whereas CCK has been shown to facilitate acetylcholine release from gallbladder neurons.[9,10] Also, the gallbladder contractile effect of CCK infusion in vivo is inhibited by atropine.[11] Evidence for the presence of intrinsic cholinergic nerves of the gallbladder has been provided by immunohistochemical studies[12] and by EFS, which stimulates intrinsic nerves, causing release of acetylcholine.[13,14] The acetylcholine released from these intrinsic nerves acts on smooth muscle muscarinic receptors causing gallbladder contraction. Our present study demonstrates that EFS is impaired in chronic acalculous cholecystitis, which suggests another mechanism for decreased gallbladder emptying in this disease.

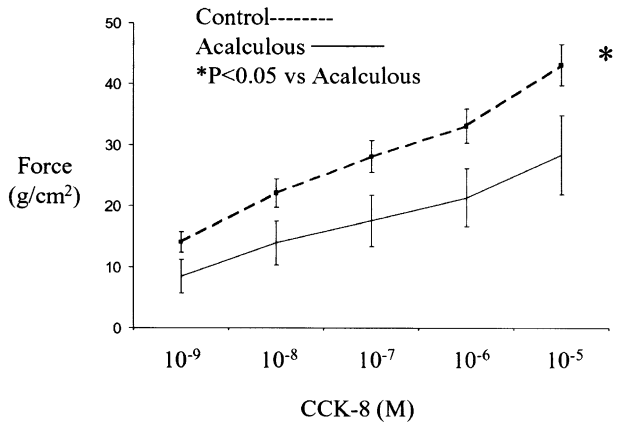


Fig. 3. Dose-response studies with CCK-8 in gallbladder smooth muscle strips from control and chronic acalculous cholecystitis gallbladder specimens. Contractions were decreased in muscle strips from gallbladders with chronic acalculous cholecystitis compared to controls. Values are means ± SEM of two experiments. *$P < 0.05$ vs. control; ANOVA.

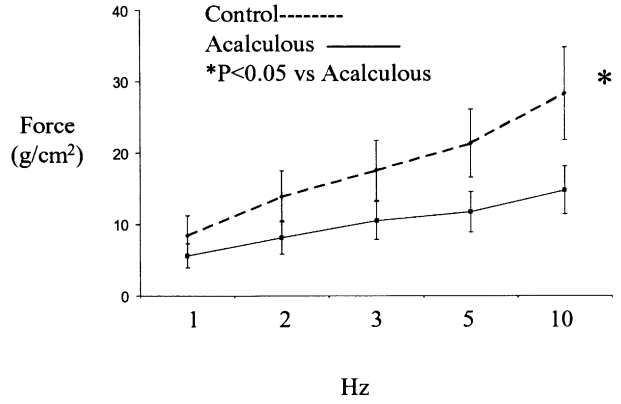


Fig. 4. Nerve-mediated contractile response to EFS in gallbladder smooth muscle strips from control and chronic acalculous cholecystitis gallbladder specimens. Contractions were decreased in muscle strips from gallbladders with chronic acalculous cholecystitis compared to controls. Values are mean ± SEM of two experiments. *$P < 0.05$ vs. control; ANOVA.

Previous experimental preparations of acalculous cholecystitis have also demonstrated similar impaired gallbladder contractility. Our laboratory recently demonstrated that endotoxin decreases the contractile response to CCK-8 for up to 96 hours and decreases the contractile response to EFS for 48 hours.[15] However, this model simulates acute acalculous cholecystitis with accompanying gallbladder injury including disrupted mucosal surfaces, coagulation necrosis, hemorrhage, and areas of fibrin deposition consistent with an acute ischemic insult, which is quite different from the clinical scenario of chronic acalculous cholecystitis. Parkman et al.[16] have also demonstrated decreased smooth muscle contractility and decreased neurally mediated contractions in an experimental preparation of acalculous cholecystitis. In this model, which uses common bile duct ligation, gallbladder strips have a decreased response to acetylcholine and EFS, which is reversed with a nitric oxide synthase inhibitor. However, this model simulates common bile duct obstruction, which is also quite different from the clinical situation of chronic acalculous cholecystitis where the biliary tree is not obstructed.

In summary, gallbladders removed for chronic acalculous cholecystitis commonly have absent spontaneous contractile activity, a decreased response to the contractile agonist CCK, and a decreased contractile response to EFS, when compared to gallblad-

ders without disease. These mechanisms could contribute to the decreased gallbladder emptying seen in chronic acalculous cholecystitis.

REFERENCES

1. Kloiber R, Monar CP, Shaffer EA. Chronic biliary type pain in the absence of gallstones: The value of cholecystokinin cholescintigraphy. Am J Roentgenol 1992;159:509–513.
2. Barron LG, Rubio PA. Importance of accurate preoperative diagnosis and role of advanced laparoscopic cholecystectomy in relieving chronic acalculous cholecystitis. J Laparoendosc Surg 1995;5:357–361.
3. Canfield AJ, Hetz SP, Schriver JP, Servis HT, Hovenga TL, Cirangle PT, Burlingame BS. Biliary dyskinesia: A study of more than 200 patients and review of the literature. J GASTROINTESTINAL SURG 1998;2:443–448.
4. Amaral J, Xiao ZL, Chen Q, Yu P, Biancani P, Behar J. Gallbladder muscle dysfunction in patients with chronic acalculous disease. Gastroenterology 2001;120:506–511.
5. Ryan JP. Motility of the biliary tree. In Yamada T, ed. Textbook of Gastroenterology. Philadelphia: Lippincott, 1991.
6. Chen Q, Amaral J, Oh S, Biancani P, Behar J. Gallbladder relaxation in patients with pigment and cholesterol stones. Gastroenterology 1997;113:930–937.
7. Cullen JJ, Hinkhouse MM, Conklin JL. Characterization of the off response to electrical field stimulation (EFS) in gallbladder smooth muscle. J Surg Res 2000;88:8–12.
8. Fisher RS, Rock E, Malmud LS. Cholinergic effects on gallbladder emptying in humans. Gastroenterology 1985; 89:716–722.
9. Mawe GM. The role of cholecystokinin in ganglionic transmission in the guinea pig gallbladder. J Physiol 1991; 439:89–102.
10. Bauer AJ, Hanani M, Muir TC, Szurszewski JH. Intracellular recordings from gallbladder ganglia of opossums. Am J Physiol 1991;260:299–306.
11. Gullo L, Bolondi L, Priori P, Casanova P, Labo G. Inhibitory effect of atropine on cholecystokinin-induced gallbladder contraction in man. Digestion 1984;29:209–213.
12. Talmage EK, Pouliot WA, Schemann M, Mawe GM. Structure and chemical coding of human, canine and opossum gallbladder ganglia. Cell Tissue Res 1984;284:289–302.
13. Yau WM, Youther ML. Modulation of gallbladder motility by intrinsic cholinergic neurons. Am J Physiol 1984;247: 662–666.
14. Parkman HP, Pagano AP, Martin JS, Ryan JP. Electric field stimulation (EFS)-induced guinea pig gallbladder contractions: Role of calcium channels in acetylcholine release. Dig Dis Sci 1997;42:1919–1925.
15. Cullen JJ, Maes EB, Aggrawal S, Hinkhouse MM, Conklin JL, Ephgrave KS, Mitros FA. The effect of endotoxin on gallbladder motility: A model of acalculous cholecystitis. Ann Surg 2000;232:202–207.
16. Parkman HP, James AN, Bogar LJ, Bartula LL, Thomas RM, Ryan JP, Myers SI. Effect of acalculous cholecystitis on gallbladder neuromuscular transmission and contractility. J Surg Res 2000;88:186–192.

Decreased Gallbladder Response in Leptin-Deficient Obese Mice

Matthew I. Goldblatt, M.D., Deborah A. Swartz-Basile, Ph.D., Carol L. Svatek, B.S., Atilla Nakeeb, M.D., Henry A. Pitt, M.D.

Obesity is a major risk factor for gallstone formation, but the pathogenesis of this phenomenon remains unclear. Human data on gallbladder emptying are conflicting, and no animal data exist on the effect of obesity on gallbladder motility. Leptin, a hormone produced by adipocytes, is known to have central effects on neuropeptide Y and cholecystokinin, but the influence of leptin on the biliary effects of these hormones is unknown. Therefore we tested the hypothesis that leptin-deficient C57BL/6J-*lep*^{ob} obese mice would have decreased gallbladder responses to excitatory stimuli. Twelve-week-old lean control (C57BL/6J) (n = 22) and C57BL/6J-*lep*^{ob} obese (n = 20) female mice were fed a nonlithogenic diet. The mice were fasted overnight and underwent cholecystectomy. Whole gallbladders were placed in 3 ml muscle baths. After optimal length was determined with acetylcholine (10^{-5} mol/L, responses to increasing doses of neuropeptide Y (10^{-8} to 10^{-6} mol/L) and cholecystokinin-8 (10^{-10} to 10^{-7} mol/L) were measured. Student's *t* test and two-way analysis of variance were used where appropriate. Results were expressed as Newtons per cross-sectional area. The lean control mice had significantly greater excitatory responses to acetylcholine than the obese mice (0.37 ± 0.05 vs. 0.16 ± 0.02, $P < 0.01$). The gallbladder responses were also greater when mice were treated with neuropeptide Y (10^{-8} mol/L: 0.00 ± 0.00 vs. 0.00 ± 0.00, NS; 10^{-7} mol/L: 0.12 ± 0.02 vs. 0.05 ± 0.01, $P < 0.01$; 10^{-6} mol/L: 0.26 ± 0.08 vs. 0.06 ± 0.01, $P < 0.01$) and cholecystokinin (10^{-10} mol/L: 0.27 ± 0.04 vs. 0.13 ± 0.02, $P < 0.01$; 10^{-9} mol/L: 0.59 ± 0.08 vs. 0.27 ± 0.04, $P < 0.01$; 10^{-8} mol/L: 0.80 ± 0.11 vs. 0.37 ± 0.05, $P < 0.01$; 10^{-7} mol/L: 0.86 ± 0.11 vs. 0.44 ± 0.06, $P < 0.01$). These data suggest that genetically obese, leptin-deficient mice have decreased responses to acetylcholine, neuropeptide Y, and cholecystokinin. We conclude that decreased gallbladder motility contributes to the increased incidence of gallstones associated with obesity. (J GASTROINTEST SURG 2002;6:438–444.)

KEY WORDS: Leptin, gallbladder motility, acetylcholine, neuropeptide Y, cholecystokinin

Cholesterol gallstone disease remains a major health care problem in Western countries. Gallstones lead to significant morbidity and cost more than $6 billion annually in the United States.[1] The risk factors for cholesterol gallstones include female sex, age, parity, and obesity. Recent human data have shown that obesity is a major risk for gallstones, second only to female sex.[2] The pathogenesis of cholesterol gallstones is multifactorial with the classic triad being supersaturation of bile with cholesterol, cholesterol crystal pronucleators, and gallbladder bile stasis.[3]

Leptin is a hormone produced by fat cells that is transported into the central nervous system where it influences a number of neurotransmitters including neuropeptide Y (NPY) and cholecystokinin (CCK) primarily in the hypothalamus.[4] Leptin has been shown to affect metabolism, satiety, and activity.[5] This laboratory has recently demonstrated that leptin-deficient mice (C57BL/6J-*lep*^{ob}) have increased resting gallbladder volume, an indication of gallbladder stasis.[6] In addition, leptin-deficient mice have increased cholesterol crystal pronucleators evidenced by a decrease in the crystal observation time without

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (oral presentation).

From the Department of Surgery (M.I.G., D.A.S.-B., C.L.S., A.N., H.A.P.), Medical College of Wisconsin, Milwaukee, Wisconsin.

Supported by grant RO1-DK44279-07 from the National Institutes of Health.

Reprint requests: Henry A. Pitt, M.D., Medical College of Wisconsin, 9200 West Wisconsin Ave., Milwaukee, WI 53226. E-mail: hapitt@mcw.edu

Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(01)00046-4

a change in the cholesterol saturation index.[7] However, the effects of obesity on gallbladder motility have not been studied in these genetically obese, leptin-deficient mice. Therefore we tested the hypothesis that leptin-deficient obese mice would have decreased gallbladder responses to excitatory stimuli compared to lean control mice when fed a nonlithogenic chow diet.

MATERIAL AND METHODS

Animals and Diet

Eight-week-old C57BL/6J lean control female mice (n = 22) (The Jackson Laboratory, Bar Harbor, Maine) and leptin-deficient obese mice (C57BL/6J-*lep*^{ob}) (n = 20) were housed four to five per cage in a light (6 AM to 6 PM)– and temperature (22° C)–controlled room. They were fed a nonlithogenic chow diet (Ralston Purina, St. Louis, Missouri) for 4 weeks. All protocols for these animal studies were approved by the Medical College of Wisconsin Animal Care Committee.

Tissue Procurement

At 12 weeks of age, after an overnight fast with water allowed ad libitum, the mice were anesthetized first with an isoflurane-soaked gauze placed in a 2000 cm^3 glass jar followed by an intraperitoneal injection of xylazine (15 mg/kg) and ketamine (50 mg/kg). The animals then underwent laparotomy and cholecystectomy. Gallbladder bile was immediately aspirated with a 30-gauge needle and syringe. Whole gallbladders were then placed in ice-cold modified Krebs solution consisting of the following in (mmol/L): NaCl, 116.6; $NaHCO_3$, 21.9; KH_2PO_4, 1.2; glucose, 5.4; $MgCl_2$, 1.2; KCl, 3.4; and $CaCl_2$, 2.5, that had been bubbled with 95% O_2/5% CO_2 for 30 minutes.

In Vitro Muscle Bath Experiments

The gallbladders were secured at their longitudinal poles with 7-0 polypropylene sutures (Ethicon, Somerville, New Jersey). The gallbladders were mounted in a 3 ml muscle bath containing modified Krebs solution bubbled with 95% O_2/5% CO_2 and maintained at 37° C. The strips were allowed to equilibrate at 0.025 *g* tension for at least 45 minutes, exchanging solution every to 10 to 15 minutes. After equilibration, optimal length was determined by response to 10^{-5} mol/L acetylcholine (ACh) (Sigma Chemical, St. Louis, Missouri) with 0.0375 to 0.1375 *g* tension. Next, increasing concentrations of NPY (10^{-8} to 10^{-6} mol/L) and cholecystokinin-octapeptide (CCK-8; 10^{-10} to 10^{-7} mol/L) (Sigma Chemical) were added to the gallbladder muscle bath. The muscle bath chambers were rinsed with modified Krebs solution after each treatment. The gallbladders were allowed to return to baseline for at least 10 minutes before a new treatment was added. At the conclusion of the experiments, the length of the gallbladder muscle between the sutures at optimal length was measured. The gallbladder segments between the sutures were then weighed, and the cross-sectional areas were calculated. Signals were recorded on a Grass 7D polygraph with a Grass FT03C transducer (Grass Instruments, Quincy, Massachusetts). Excitatory responses were measured as magnitude from baseline and are expressed as Newtons per cross-sectional area (N/cm^2) (Fig. 1). Muscle responses were quantified using WINDAQ/EX computer software (Dataq Instruments, Inc., Akron, Ohio). EC_{50} values for CCK-8–induced excitatory responses were determined using GraphPad Prism software (GraphPad Software, Inc., San Diego, California).

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analyses were performed using SigmaStat Statistical Software (Jandel Corp., San Rafael, California). Differences in animal weights and muscle bath responses were tested for statistical significance by Student's unpaired *t* test and two-way analysis of variance as appropriate. A *P* value of <0.05 was considered statistically significant.

RESULTS

Age and Body Weight

The ages of the animals at the time of surgery did not differ between the C57BL/6J lean control mice and the C57BL/6J-*lep*^{ob} leptin-deficient obese mice (12.0 ± 0.1 weeks vs. 11.8 ± 0.1 weeks, $P = 0.14$). As expected, significant differences in final body weight were observed between the lean control mice and the leptin-deficient obese mice at the time of surgery (17.6 ± 0.3 g vs. 49.2 ± 0.7 g, $P < 0.01$).

Muscle Bath

The gallbladder cross-sectional areas from the lean mice did not differ from those of the leptin-deficient obese mice ($1.6 \times 10^{-3} \pm 9.0 \times 10^{-5}$ cm^2 vs. $1.5 \times 10^{-3} \pm 9.4 \times 10^{-5}$ cm^2, $P = 0.44$, respectively). Responses to ACh at optimal length were greater in the lean mice compared to the obese mice (Table 1). Gallbladder responses to increasing concentrations of NPY were significantly increased in the lean mice compared to the obese mice, except at the concentration of 10^{-8} mol/L (see Table 1 and Fig. 2). Excitatory responses of the gallbladders to CCK-8 were

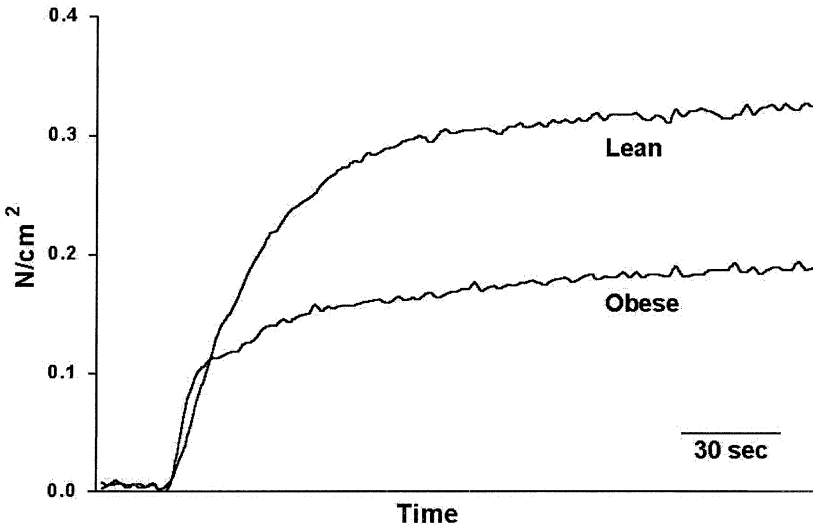


Fig. 1. Typical gallbladder responses in vitro to excitatory neurotransmitters. CCK-8 was used in this example. The responses are represented as force per unit area (N/cm^2) and are plotted over time from lean control C57BL/6J mice and leptin-deficient (C57BL/6J-*lep*^{ob}) mice.

also decreased in the obese mice compared to the lean control mice (see Table 1 and Fig. 3). The EC_{50} values of the CCK-8 concentration response curves were lower in the lean mice compared to the obese mice ($7.56 \times 10^{-10} \pm 1.34 \times 10^{-10}$ vs. $2.30 \times 10^{-9} \pm 7.97 \times 10^{-10}$, $P = 0.05$).

DISCUSSION

The three key components to cholesterol gallstone formation are cholesterol supersaturation of bile, cholesterol crystal pronucleators, and biliary stasis.[3] Biliary stasis is influenced not only by sphincter of Oddi motility and gallbladder filling but also by gallbladder emptying, which is controlled by gallbladder smooth muscle. These studies demonstrate that the response of gallbladder smooth muscle to excitatory stimuli such as ACh, NPY, and CCK in leptin-deficient obese mice is markedly reduced. In addition, the EC_{50} of CCK is significantly increased indicating that a higher concentration of CCK is required to achieve a 50% maximal response in the gallbladders of obese mice.

Obesity is a major health care issue because it affects more than 50,000,000 Americans.[8] Gallstone disease has long been associated with obesity. Nakeeb et al.[2] demonstrated in a multivariate analysis that persons with a body mass index greater than 30 had a 3.7-fold increase in the risk of gallstones. Several mechanisms have been proposed for this find-

Table 1. Muscle bath results

Treatment		Lean control	Leptin-deficient	*P* value
ACh	10^{-5} mol/L	0.37 ± 0.05	0.16 ± 0.02	<0.01
NPY	10^{-8} mol/L	0.00 ± 0.00	0.00 ± 0.00	NS
	10^{-7} mol/L	0.12 ± 0.02	0.05 ± 0.01	<0.01
	10^{-6} mol/L	0.26 ± 0.08	0.06 ± 0.01	<0.01
CCK-8	10^{-10} mol/L	0.27 ± 0.04	0.13 ± 0.02	<0.01
	10^{-9} mol/L	0.59 ± 0.08	0.27 ± 0.04	<0.01
	10^{-8} mol/L	0.80 ± 0.11	0.37 ± 0.05	<0.01
	10^{-7} mol/L	0.86 ± 0.11	0.44 ± 0.06	<0.01

Values are mean ± SEM; results are expressed as N/cm^2; NS = not significant.

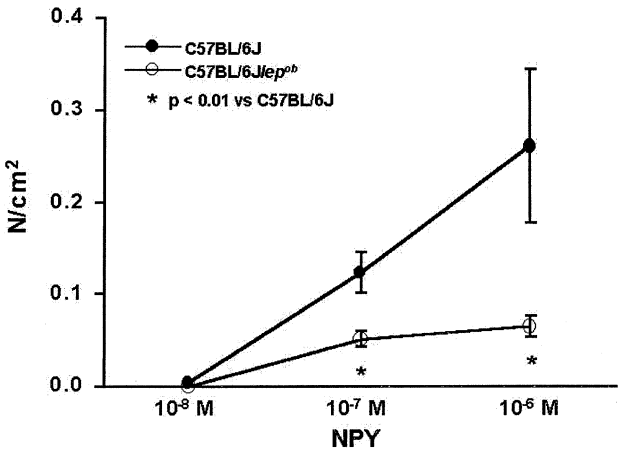


Fig. 2. Whole gallbladders from lean (C57BL/6J) and leptin-deficient obese (C57BL/6J-*lep*^{ob}) mice were mounted into 3 ml in vitro muscle baths. Various concentrations of NPY were added and the maximal excitatory responses were recorded. Responses were measured as stress (N) per unit area (cm^2). The gallbladders from the obese animals showed significantly decreased responses compared to the lean control mice. Two-way analysis of variance was used to compare results.

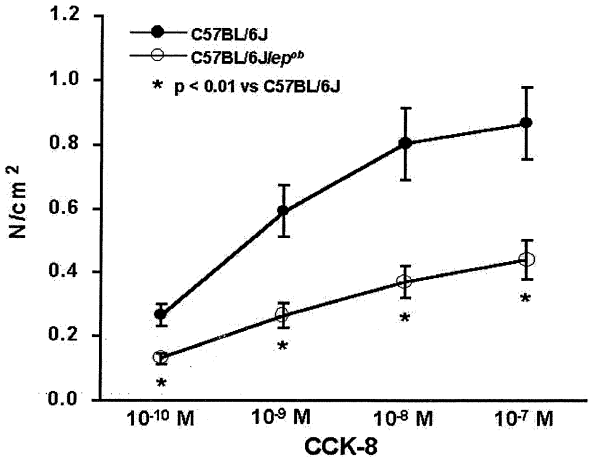


Fig. 3. Excitatory responses to various concentrations of CCK-8 were measured in whole gallbladders from lean and leptin-deficient obese mice. Responses were measured as stress (N) per unit area (cm^2). Two-way analysis of variance was used to compare results.

ing. One etiology is believed to be due to cholesterol-supersaturated bile, which is seen in patients with cholesterol gallstones.[9,10] Biliary motility has been implicated in obesity-related cholesterol gallstones, but human data are conflicting. Gallbladder resting volume, an indication of biliary stasis, has been shown to increase in proportion to fat mass in obese patients.[11] However, reports on gallbladder emptying in obesity have been conflicting. Although some studies show that obesity is associated with decreased gallbladder emptying,[12] a number of studies have failed to show that the gallbladder response to CCK or a fatty meal is impaired in the obese patient.[13,14]

Leptin is a 16 kDa peptide hormone product of the ob gene that is produced by white fat cells with receptors found primarily in the hypothalamus.[15] The leptin-deficient mouse develops morbid obesity by maturity with increased food intake and decreased energy expenditure.[16] In addition, the leptin-deficient mouse will lose weight and increase activity when the deficient leptin is replaced.[16] The actions of leptin in the brain are complex and are mediated by neurotransmitters such as NPY and CCK.[4] Not only has the connection between leptin and CCK been made in the brain, but the CCK receptor subtype CCK-B is pivotal in the regulation and release of leptin from fat cells in rats.[17] Because of the multiple interactions between leptin and known stimulators of gallbladder smooth muscle, the leptin-deficient mouse was employed in this study as a model for obesity.

Intrinsic cholinergic nerves are important for gallbladder emptying in vivo. Human patients who have received atropine have demonstrated decreased gallbladder emptying after a test meal.[18] Parkman et al.[19] demonstrated that the M_3 subtype of the muscarinic receptor was responsible for direct gallbladder smooth muscle stimulation in guinea pigs. These investigators also showed that M_1 and M_2 type muscarinic receptors were on cholinergic nerves and act as prejunctional autoreceptors in the gallbladder. In the present study, acetylcholine stimulated weaker contractions within the gallbladders of obese mice. Further studies would need to be performed to determine whether the excitatory response is due to prejunctional or direct smooth muscle receptors, and also which muscarinic receptors are involved in the mouse.

The role of NPY in the central nervous system to control food intake and its relationship with leptin has been well described.[20] In the leptin-deficient ob mouse and the leptin-unresponsive db mouse, hypothalamic levels of NPY are elevated.[21] Also, administration of NPY into cerebral ventricles induces significant feeding behavior.[22] In the periphery, NPY was unable to elicit a direct effect on rat anococcygeus muscle, but instead appeared to inhibit the relaxation caused by nonadrenergic, noncholinergic neurons.[23] NPY does have direct excitatory effects in the biliary tree by stimulating gallbladder contractions and sphincter of Oddi phasic wave contractions in the prairie dog.[24] The current study demonstrated two findings with NPY. First, NPY caused an excitatory response in the gallbladder smooth muscle. Second, the excitatory response was diminished in obesity. The NPY receptors Y1 and Y2 have been implicated in peripheral excitatory effects.[25] The exact mechanism in this current model will require further description.

CCK and leptin have been shown to act synergistically in the central nervous system by reducing food intake in experimental animals.[26] CCK receptors have been extensively studied in the context of gallbladder motility related to gallstone disease. We demonstrate that CCK-induced gallbladder contraction is decreased in leptin-deficient obese mice fed a nonlithogenic diet. None of these animals have cholesterol-saturated bile, cholesterol crystals, or gallstones, indicating that gallbladder dysmotility precedes the formation of stones and is not the result of gallstones. This finding is not likely to be the result of a mutation of CCK receptors, as Nardone et al.[27] demonstrated that the CCK receptor gene shows no evidence of sequence mutation in patients with either gallbladder disease or obesity.[27] Despite the normal sequence of the CCK receptor, its dysfunction has been associated with the decreased contractile response seen in patients with cholesterol gallstones.[28] However, this receptor dysfunction is reversed

when the tissues are relieved of excess membrane cholesterol.

Bile cholesterol has been shown to diffuse readily into the smooth muscle plasma membranes,[29] but increased serum cholesterol does not seem to have the same effect.[28] We have previously shown that leptin-deficient obese mice have bile cholesterol levels that are similar to those in lean control mice and, therefore, the gallbladder smooth muscle would not be expected to have a different cholesterol content.[6] In the present study the decreased excitatory response in the obese mice was most likely due to second messengers further downstream from the CCK receptor. Indeed, bypassing the CCK receptor by directly stimulating downstream G proteins pharmacologically returns the contractile response of the gallbladder smooth muscle from patients with cholesterol gallstones to that of pigment gallstone control mice.[30] Future studies in the leptin-deficient mouse would include verifying the cholesterol content of the gallbladder smooth muscle and directly stimulating G proteins to determine whether the decreased contractile response of the obese mice returns to baseline.

These experiments demonstrate that a murine model can be used to examine gallbladder motility. In addition, the clinical finding of decreased gallbladder motility in obese humans has been duplicated in this model of obesity. In conclusion, this study suggests that decreased gallbladder motility plays a role in the pathogenesis of gallstone formation in obesity and that leptin may be involved in this phenomenon.

REFERENCES

1. Graves EJ, Owings MF. 1995 Summary: National Hospital Discharge Survey. Advance Data From Vital and Health Statistics; No. 291. National Center for Health Statistics 1997.
2. Nakeeb A, Goldblatt MI, Swartz-Basile DA, et al. The role of genetics in gallstone pathogenesis. Gastroenterology (in press).
3. Carey MC. Critical tables for calculating the cholesterol saturation of native bile. J Lipid Res 1978;19:945–955.
4. Inui A. Feeding and body-weight regulation by hypothalamic neuropeptides—mediation of the actions of leptin. Trends Neurosci 1999;22;62–67.
5. Zhang Y, Proenca R, Maffei M, et al. Positional cloning of the mouse obese gene and its human homologue. Nature 1994;372:425–432.
6. Swartz-Basile D, Choi S-H, Blaser C, et al. Gallbladder volume and composition in genetically obese mice. Gastroenterology 2000;118:12.
7. Goldblatt M, Choi S-H, Swartz-Basile D, et al. Cholesterol crystal formation in congenitally obese mice. Surg Forum 2000;51:1–2.
8. Mokdad A, Serdula M, Dietz W, et al. The spread of the obesity epidemic in the United States, 1991–1998. JAMA 1999;282:1519–1522.
9. Shaffer EA, Small DM. Biliary lipid secretion in cholesterol gallstone disease. The effect of cholecystectomy and obesity. J Clin Invest 1977;59:828–840.
10. Amaral JF, Thompson WR. Gallbladder disease in the morbidly obese. Am J Surg 1985;149:551–557.
11. Hendel HW, Hojgaard L, Andersen T, et al. Fasting gall bladder volume and lithogenicity in relation to glucose tolerance, total and intra-abdominal fat masses in obese non-diabetic subjects. Int J Obes 1998;22:294–302.
12. Palasciano G, Portincasa P, Belfiore A, et al. Gallbladder volume and emptying in diabetics: The role of neuropathy and obesity. J Intern Med 1992;231:123–127.
13. Vezina WC, Paradis RL, Grace DM, et al. Increased volume and decreased emptying of the gallbladder in large (morbidly obese, tall normal, and muscular normal) people. Gastroenterology 1990;98:1000–1007.
14. Acalovschi M, Badea R. Ultrasonographic study of gallbladder emptying in obese patients. Int J Obes Relat Metab Disord 1992;16:313–315.
15. Yanovski JA, Yanovski SZ. Recent advances in basic obesity research. JAMA 1999;282:1504–1506.
16. Halaas JL, Gajiwala KS, Maffei M, et al. Weight-reducing effects of the plasma protein encoded by the obese gene. Science 1995;269:543–546.
17. Attoub S, Levasseur S, Buyse M, et al. Physiological role of cholecystokinin B/gastrin receptor in leptin secretion. Endocrinology 1999;140:4406–4410.
18. Fisher RS, Rock E, Malmud LS. Cholinergic effects on gallbladder emptying in humans. Gastroenterology 1985; 89:716–722.
19. Parkman HP, Pagano AP, Ryan JP. Subtypes of muscarinic receptors regulating gallbladder cholinergic contractions. Am J Physiol 1999;276:1243–1250.
20. Rohner-Jeanrenaud E, Jeanrenaud B. Central nervous system and body weight regulation. Ann Endocrinol 1997; 58:137–142.
21. Wilding JP, Gilbey SG, Bailey CJ, et al. Increased neuropeptide-Y messenger ribonucleic acid (mRNA) and decreased neurotensin mRNA in the hypothalamus of the obese (ob/ob) mouse. Endocrinology 1993;132:1939–1944.
22. White BD, Martin RJ. Evidence for a central mechanism of obesity in the Zucker rat: Role of neuropeptide Y and leptin. Proc Soc Exp Biol Med 1997;214:222–232.
23. Vila E, Tabernero A, Fernandes F, Salaices M. Effect of neuropeptide Y on adrenergic and non-adrenergic, non-cholinergic responses in the rat anococcygeus muscle. Br J Pharmacol 1992;107:66–72.
24. Lillemoe KD, Webb TH, Pitt HA. Neuropeptide Y: A candidate neurotransmitter for biliary motility. J Surg Res 1988;45:254–260.
25. Hoyo Y, McGrath JC, Vila E. Evidence for Y1-receptor-mediated facilitatory, modulatory cotransmission by NPY in the rat anococcygeus muscle. J Pharmacol Exp Ther 2000;294:38–44.
26. Barrachina MD, Martinez V, Wang L, et al. Synergistic interaction between leptin and cholecystokinin to reduce short-term food intake in lean mice. Proc Natl Acad Sci U S A 1997;94:10455–10460.
27. Nardone G, Ferber IA, Miller LJ. The integrity of the cholecystokinin receptor gene in gallbladder disease and obesity. Hepatology 1995;22:1751–1753.
28. Xiao ZL, Chen Q, Amaral J, et al. CCK receptor dysfunction in muscle membranes from human gallbladders with cholesterol stones. Am J Physiol 1999;276:G1401–G1407.
29. Ross PE, Butt AN, Gallacher C. Cholesterol absorption by the gall bladder. J Clin Pathol 1990;43:572–575.
30. Yu P, Chen Q, Harnett KM, et al. Direct G protein activation reverses impaired CCK signaling in human gallbladders with cholesterol stones. Am J Physiol 1995;269:659–665.

Discussion

Dr. K. Lillemoe (Baltimore, MD.): As you stated, the hormone leptin has been primarily shown to have effects in the central nervous system. Is there any reason to think that this hormone has an effect at the gallbladder muscle level? Have receptors for leptin been identified in the gallbladder?

Second, which sort of follows along the same line of thinking, is this a "chicken or egg" phenomenon? In other words, in previous studies you noted in these same leptin-deficient animals that their gallbladders have an increased gallbladder volume, which suggests stasis. Therefore, when you remove the gallbladder from the animal and place it in your ex vivo chamber, are you just seeing a secondary effect, that is, has the dilated, distended gallbladder lost its responsiveness at the muscular level?

Third, with Dr. Nakeeb's expertise in morbid obesity, you most likely have access to gallbladders that were removed from patients undergoing surgery for morbid obesity. Have you studied human gallbladders from patients with morbid obesity in this model?

Dr. M. Goldblatt: With regard to leptin and looking at leptin receptors throughout the gastrointestinal tract, there have been studies demonstrating the influence of leptin and CCK in the stomach. We have shown, at least with some preliminary findings, that there are leptin receptors in the gallbladders of humans. As far as how leptin relates to our findings, that remains to be sorted out.

As to the actual mechanism in the muscle bath in the gallbladders and whether or not they have just been overstretched, one of the things we do as soon as the gallbladder is removed is drain the bile, and these gallbladders are allowed to rest for at least a couple of hours before any of the experiments take place. That is not to say that if they are overstressed, that is enough time for them to recover, but at least that is part of it. Some of our preliminary in vivo data have shown that these gallbladders, when given enough stimulation, do seem to have the ability to contract fully.

Finally, in the human scenario, we have started to look at humans, both obese subjects, as you mentioned, and some lean control subjects. The preliminary data are very much in the infancy stage, so we really do not have sufficient numbers to comment.

Dr. D. Dempsey (Philadelphia, PA): I would like to compliment you on some very elegant studies. I would say on behalf of those of us who try to interpret muscle bath data, that your results show very impressive differences in these leptin-deficient animals. In most of the obese patients that you and I operate on, is the leptin level low or high?

Dr. Goldblatt: Most humans have increased leptin levels. As opposed to a deficiency in leptin, it seems that they have a problem with the leptin receptor being insensitive to leptin. So in that sense this model does not mimic responses in the majority of obese patients. There are models of obesity in mice, such as the db mouse, which actually has a problem with the leptin receptor, that we are going to use in addition to this model to help examine whether or not this is a leptin or a leptin receptor problem.

Dr. M. Dayton (Salt Lake City, UT): I realize that this study was primarily a motility study and an examination of neurotransmitters, but I am interested in knowing whether or not your model, the obese model, actually forms gallstones? In other words, when fed a lithogenic or even a nonlithogenic diet, do these obese mice form gallstones? It may give us some idea of how good a model this would be for predicting the effect of neurotransmitters.

Dr. Goldblatt: The background strain that we chose for this model, the C57BL/6J mouse, has been shown to actually have an increased propensity for gallstone formation. We chose this background strain so that if we saw anything after adding the obesity factor, it should be above and beyond the lean control. What we have shown when we have fed them a lithogenic diet is that they have an increased number of cholesterol crystals after 4 weeks. We fed them only out to 4 weeks. We have not gone further to see if there are actual gallstones, but there definitely is a difference in the number of crystals.

Invited Discussion—Expert Commentator

Lawrence W. Way (San Francisco, CA): The paper about the gallbladder response to leptin leaves me with not much to say, so maybe I shouldn't say anything. The only reaction I had is why couldn't this experiment have

been done just as well in humans? I harken back to my college days and would say that the authors' final slide, although it is nice to be optimistic and upbeat about things, probably reflected a little example of hasty generalization as the logical fallacy there, because the conclusion was that this means there is a motor defect in the gallbladder in obese patients. I am not sure the model can carry us that far.

Age-Associated Changes in Gene Expression Patterns in the Liver

Robert P. Thomas, M.D., Michelle Guigneaux, M.S., Thomas Wood, Ph.D., B. Mark Evers, M.D.

Aging is one of the least clearly understood biological processes. Alteration of oxidation/reduction (redox) enzymes has been demonstrated with aging; however, a systematic analysis of expression patterns has not been performed. The liver plays a key role in homeostasis and detoxification; therefore alteration of hepatic gene expression with aging may affect outcome after surgery. The purpose of our study was to assess changes in gene expression patterns in aged livers from both rats and humans using gene array analysis. Total RNA was extracted from young (2-month-old) and aged (2-year-old) rat livers, as well as young (1-year-old) and aged (78-year-old) human livers. Gene expression patterns were compared using Affymetrix GeneChip arrays. The expression pattern of selected genes was confirmed by reverse transcription–polymerase chain reaction. A threefold or greater change in gene expression was noted in 582 genes in the aged rat livers and 192 genes in the aged human livers. Comparison of the genes that were increased with aging demonstrated some similar patterns of expression in the rat and human livers, particularly in members of the antioxidant family and the cytochrome P-450 genes. Our findings demonstrate changes in the expression pattern of genes in the liver with aging. Concomitant increases in the expression of important antioxidant and detoxifying genes were noted in the livers of both rats and humans. This induction pattern suggests a complex link between changing hepatic detoxification/redox capability and senescence. (J GASTROINTEST SURG 2002;6:445–454.)

KEY WORDS: Aging, gene expression, liver, gene array

Medical advances in the past century have enabled persons in our society to live longer and remain healthy for a longer period of time. It is estimated that the number of elderly will continue to increase and by the year 2050, nearly 25% of Americans will be at least 65 years of age.[1] As the number of persons reaching advanced age increases, there will be an associated need for increased surgical care. From 1980 to 1996, the percentage of operations that were performed in patients over 65 years of age increased from 19% to 36%.[2] Furthermore, it is estimated that at least 50% of patients in most general surgery practices are over 65 years of age.[3] With aging comes a decline in physiologic function in most organ systems.[4] In normal, nonstressful conditions, this decline usually has minimal consequences; however, with stress (e.g., surgery or trauma), the ability of the aged patient to respond to these increased demands is seriously compromised.[5] Therefore it is imperative that all physicians recognize and understand the changes that occur in various organ systems with aging, if we are to achieve improvements in the care of elderly patients.

Aging, a universal phenomenon of all organisms, is one of the least clearly understood biological processes. There are currently a number of theories proposed to explain the changes associated with aging.[6] Oxidative damage to mitochondrial DNA is thought to play a role in aging and in various degenerative processes.[7] This theory, often called the free-radical theory of aging, provides a conceptual approach to the study of aging.[8–10] Free radicals are reactive oxy-

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (oral presentation).

From the Department of Surgery (R.P.T., B.M.E.) and The Molecular Genomics Core Laboratory (M.G., T.W.), The University of Texas Medical Branch, Galveston, Texas.

Supported by grants P01 DK35608, R01 AG10885, and T32 DK07639 from the National Institutes of Health. Dr. Thomas is the recipient of a Jeane B. Kempner Scholar Award.

Reprint requests: B. Mark Evers, M.D., Department of Surgery, The University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-0536. e-mail: mevers@utmb.edu

1091-255X/02/$—see front matter
PII: S1091-255X(01)00010-5

gen species (ROS) that are normally generated at low levels in all living aerobic cells by the respiratory chain of the mitochondria. The accumulation of ROS is thought to damage cellular machinery and contribute to the changes seen with aging.[8] To oppose this internal threat, cells have a number of antioxidant defense mechanisms including antioxidant enzymes such as superoxide dismutase (SOD), which catalyzes the dismutative reaction between two superoxide molecules and hydrogen to form H_2O and O_2.[11] Other antioxidants include the glutathione S-transferase (GST) enzymes, which eliminate ROS through conjugation reactions and NADH–cytochrome b_5 reductase, which reduces the membrane antioxidant coenzyme Q.[12] These enzymes provide a scavenging system to eliminate potentially harmful oxygen metabolism byproducts, but it is estimated that the average human cell still encounters approximately 10,000 free-radical exposures a day.[13]

The liver is a critical organ in protection from oxidative damage.[14] In addition, the liver plays a major role in the breakdown of potentially toxic lipophilic toxins through the action of oxidation and reduction enzymes such as the cytochrome P-450 enzymes.[15] This family of enzymes adds hydroxyl groups to a number of toxins, which facilitates their excretion in the urine.[16] Age-associated changes in liver physiology and function have been described.[17] With aging, there is a general decrease in organ size and hepatocyte number. Liver enzymes are routinely unaltered with senescence; however, during periods of stress, the liver in the aged patient may not be able to meet the demands of hypermetabolic states. Elucidating the changes in the expression of genes in the liver with aging may lead to a better understanding and treatment of hepatic dysfunction after surgery or major stress. Therefore the purpose of our study was to assess age-associated changes in gene expression patterns in rat livers and compare them with changes in human livers. We used gene array technology for these studies. Genomic expression profiling with high-density oligonucleotide arrays represents a powerful tool to expand current molecular studies and provide a global assessment of changes in gene expression patterns associated with aging.

MATERIAL AND METHODS

Total RNA was isolated using Ultraspec II (Biotex, Houston, TX). GeneChip oligonucleotide glass-slide, high-density arrays and hybridization protocols were obtained from Affymetrix (Santa Clara, CA) (rat array: RG_U34A = 8,797 gene probes; human array: HG_U95A = 12,626 gene probes). In addition, Affymetrix GeneChipSuite 3.3 expression analysis software and Affymetrix EASI databases were used. Decision Site Gene Array Explorer software was also purchased. Polymerase chain reaction (PCR) reagents were purchased from GIBCO-BRL (Grand Island, NY), and PCR primers were synthesized by Oligos Etc. (Wilsonville, Ore.). For the GST activity assay, the substrate, 1-chloro-2,4-dinitrobenzene, and glutathione were purchased from Sigma Chemical (St. Louis, MO). All other reagents were of molecular biology grade and were purchased from either Sigma or Amresco (Salon, OH).

Animals and Tissue Preparation

Young (2-month-old) and aged (24-month-old) male Fischer 344 rats were obtained from the National Institute on Aging (Bethesda, MD) stock colony maintained under barrier-reared conditions. Aged animals were defined as those that had achieved the age at which one-half of the population ordinarily dies (median survival time). In humans that age is approximately the eighth decade of life; in Fischer 344 rats the age is 23 to 26 months.[18] After receipt, rats were housed at a constant temperature of (22° C) with 12-hour light-dark cycles. Rats were fed standard rat chow (Ralston Purina, St. Louis, MO) ad libitum.

At the end of a 7-day acclimatization period, the rats were anesthetized with 5% halothane and then killed by decapitation. The entire liver was removed, immediately frozen in liquid N_2, and maintained at −70° C until RNA extraction. Two human liver samples from a 1-year-old male (young) and a 78-year-old male (aged), both of whom sustained intracranial injuries, were purchased from Tissue Transformation Techniques (Edison, NJ). Tissue samples were stored at −70° C until extracted for RNA. Tissue acquisition and subsequent use were approved by the Institutional Review Board at The University of Texas Medical Branch.

RNA Isolation and Affymetrix GeneChip Labeling Analysis

For both rat and human samples, total RNA was extracted by the method of Chomczynski and Sacchi[19] using Ultraspec RNA in accordance with the manufacturer's instructions.

Using total RNA (25 to 30 μg), first-strand cDNA was synthesized with a T7 - $(dT)_{24}$ oligomer (5′ GGCCAGTGGAATTGTAATA CGACTCACTA-TAGGGAGGCGG-dT_{24} 3′) and SuperScript II reverse transcription (RT; Life Technologies, Rock-

ville, MD). Second-strand synthesis converts the cDNA into the DNA template needed for in vitro transcription. During in vitro transcription, target RNAs (cRNA) were biotin labeled with bacteriophage T7 RNA polymerase-directing synthesis. The biotin-labeled cRNAs then were fragmented to a mean size of 150 bases. The samples were tested with hybridization to test chips containing several housekeeping genes (e.g., actin, GAPDH).

Hybridizations were performed at 45° C for 16 hours in 0.1 mol/L MES (pH 6.6), 1 mol/L NaCl, 0.02 mol/L EDTA, and 0.01% Tween 20. Four prokaryotic genes (*bio* B, *bio* C, *vio* D from *E. coli* biotin synthesis pathway, and *cre*, the recombinase gene from P1 bacteriophage), were added to the hybridization cocktail as internal controls. These control RNAs were used to normalize expression levels between experiments and, because they are added at varying copy number (*Bio* B, 1.5 pmol/L; *Bio* C, 5 pmol/L; *Bio* D, 25 pmol/L and CRE, 100 pmol/L), they were used in estimating relative abundance of RNA transcripts in the sample. The arrays (rat: RG_U34A, 8,799 genes; human HG_U95A, 12,626 genes) were washed with nonstringent (1 mol/L NaCl, 25° C) and stringent (1 mol/L NaCl, 50° C) solutions. Staining was completed with phycoerythrin streptavidin (10 mg/ml final). The GeneChips were then scanned with a Hewlett-Packard scanner and analyzed. Further data analysis was performed using the Affymetrix EASI database (to assign gene descriptions to query probe sets) and the Spotfire Decision Site software system (Somerville, Mass.).

Reverse Transcription–Polymerase Chain Reaction

For RT-PCR, the sequences of the specific primers used for the reactions were as follows: rat NADH–cytochrome b5 reductase, (sense) 5′ ATCCCAAGT TTCCAGCTGGAG-3′ and (antisense) 5′-CCAGTACAGCTGGATGACCAG-3′; rat Cu/Zn SOD, (sense) 5′-GGCGGCTTCTCTCGT CTCCTTG-3′ and (antisense) 5′CAGCAGATGATGAGTCTGAGACTCAG-3′; human cytochrome P-450 1A1 (TCCD inducible), (sense) 5′CAGTAATGGTCAGAGCATGTCC-3′ and (antisense) 5′-GGTTCACACCAAATACATGAGGCT-3′; human cytochrome P-4502C18 (CYP2C18), (sense) 5′-GGTAACAAAGACTTGGAATCC-3′ and (antisense) 5′-GCAGGAGTCCATATCTCAG-3′; and glyceraldehyde-3-phosphatase dehydrogenase (GAPDH), (sense) 5′-TCCACCACCTGTTGCTGTA-3′ and (antisense) 5′-ACCACAGTCCATGCCATGCCATCAC-3′.

The RT reaction was performed according to the manufacturer's instructions (GIBCO-BRL) and as previously described.[20] Briefly, a 50 μl reaction consisting of 5 μg total RNA, 1 μl oligo (dT) and RNase free water was incubated for 10 minutes at 70° C. The mixture was then mixed with 5× buffer, 0.1 mol/L DTT, and 10 mmol/L dNTP mix and incubated for 2 minutes at 42° C. This was followed by the addition of SuperScript II (200 U/μl) and further incubated for 50 minutes at 42° C. The reaction was halted with a temperature increase to 70° C for 15 minutes. A parallel reaction without the addition of RNA was performed to ensure lack of contamination.

After the completion of the RT reactions, 1 μl of cDNA template was used for PCR. Reaction mixtures contained 1 μl of cDNA, 5 μl of 10× PCR buffer, 1.5 μl of 50 mmol/L MgO_2, 1 μl of 10 nmol/L dNTP, and 1 μl of 10 μmol/L sense and antisense primers. Autoclaved-distilled H_2O was added to a total volume of 50 μl. Reactions were mixed and covered with sterile mineral oil, and PCR was initiated with 95° C hot-start addition of Taq DNA polymerase and carried out according to the following profile: an initial denaturation for 1 minute at 95° C followed by 25 cycles at 94° C for 55 seconds, 60° C for 55 seconds, and 72° C for 1 minute and 30 seconds. Additional parallel reactions were performed without the addition of cDNA template to ensure lack of contamination. An aliquot of each PCR reaction was resolved by gel electrophoresis with 1% agarose gel containing ethidium bromide. Bands were visualized by ultraviolet illumination.

Glutathione S–Transferase Activity Assay

Total GST activity of cytosolic fractions of protein was assayed using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate as described originally by Habig et al.[21] Frozen liver samples were homogenized in a lysis buffer (0.25 mol/L sucrose, 1 mmol/L EDTA, pH 7.4). Cytosolic fractions were obtained by centrifugation (14,000 rpm for 30 minutes, followed by 45,000 rpm for 60 minutes). Protein concentration was determined by the method of Bradford.[22] Reaction mixtures, consisting of 1.5 ml potassium buffer (0.13 mol/L, pH 6.5), 20 μl CDNB (76 mg/10 ml ETOH), and 10 μl protein sample (1:10 dilution), were analyzed by spectrophotometry at a wavelength of 340 nm after the addition of GSH (76 mg/10 ml H_2O), which initiates enzyme activity.[23] Activity measurements were standardized to protein content and expressed as μmol/min/mg protein.

Statistical Methods

Rat GST activity per minute was analyzed using analysis of variance for a two-factor experiment with

repeated measures on time. The two factors were age group (2 and 24 months) and time (5 points from 1 to 5 minutes). Effects and interaction were assessed at the 0.05 level of significance. Fisher's least significant difference procedure was used for multiple comparisons with Bonferroni adjustment for the number of comparisons.

RESULTS

Age-Associated Changes in the Expression Pattern of Genes in Rat and Human Livers

To determine the effects of aging on the expression pattern of genes in the liver, we extracted RNA from young and aged rat and human livers; gene expression profiles were determined using rat and human microarrays from Affymetrix. Expression changes were reflected in the differences in signal intensity from array hydrizations. Analysis of the microarray differences in the hybridization signals was accomplished with the use of Affymetrix Expression Suite 3.3 software; a threefold or greater change in signal intensity was considered a significant change in expression (Fig. 1). Analysis of expression patterns in aged rat livers demonstrated a threefold or greater change in the expression of 582 genes compared with the livers of the young animals (552 genes were increased and 30 genes were decreased with aging) (see Fig. 1, *A*). Genes with changes of threefold or greater comprised approximately 6% of the probes on the rat RG_U34A microarray, with changes ranging from a 105-fold increase to a 261-fold decrease in the expression of genes in the aged livers.

Analysis of the human (young and old) liver samples demonstrated threefold or greater changes in the expression of 192 genes (approximately 1% of the probes on the human U95A microarray) (see Fig. 1, *B*). In contrast to the rat liver in which the majority of gene changes were noted to be an actual increase, only 43 genes in the aged human liver demonstrated a threefold or greater increase of expression, whereas 149 genes were decreased by threefold or greater. Taken together, our analysis of rat and human livers has identified a subset of genes that appear to be altered with aging. A number of genes were increased threefold or greater, particularly in the rat liver, further emphasizing the fact that gene expression does not remain static or uniformly decline with senescence.

Analysis of the genes with increased expression in the aged rat and human livers demonstrated that a number of these genes are members of the redox and detoxification gene families. These included members of the GST, cytochrome P-450, UDP-glucuronosyltransferase, and SOD families, with increases of expression ranging from three- to 61-fold in the aged rat liver (Table 1). Similarly, increases in members of the GST, cytochrome P-450, and UDP-glucuronosyltransferase gene families were detected in the aged human liver (Table 2).

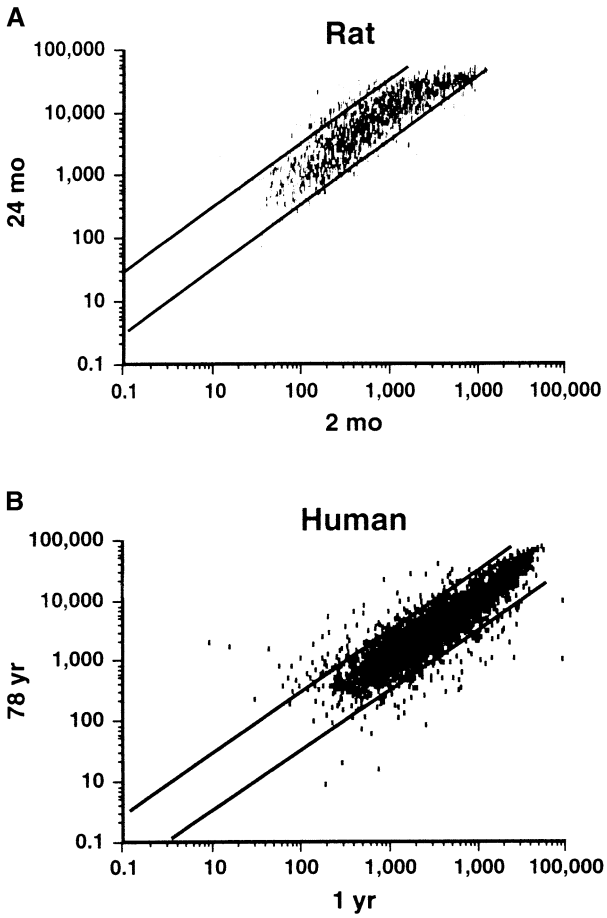


Fig. 1. Gene array hydrization signal intensities. Hydrization differences as calculated and normalized by Affymetrix Expression Suite 3.3 software. **A,** Scatter plot of differential signal intensity between young (2-month-old) and aged (24-month-old) rat livers as analyzed by RG_U34A Affymextrix GeneChip array. **B,** Scatter plot of differential signal intensity between young (1-year-old) and aged (78-year-old) human livers as analyzed by HG_U95A Affymextrix GeneChip array. Normalization for both rat and human experiments included elimination of genes with insufficient hydrization for analysis as determined by Expression Suite 3.3 software.

Confirmation of Gene Expression Changes by RT-PCR

To confirm our findings obtained by microarray analysis, the expression of selected genes was analyzed by a semiquantitative RT-PCR procedure using the same RNA samples that were used in the microarray analysis (Fig. 2). Two genes in the rat that

Table 1. Subsets of genes that are increased in the aged rat liver

Rat genes	Gene bank	Fold increase
UDP-glucoronosyltransferase		
Exon 1	D38065	29
3-Methylchoanthrene-inducible	S56937	20
UGT1	D83796	3
Glutathione S–transferase		
Subunit 13 (GSTK-1)	S83436	61
Yc2 subunit (GSTA5)	S82820	51
Subunit 8	X62660	27
P subunit	X02904	4
Cytochrome P-450 enzymes		
P-450 polypeptide	M14775	13
P-450 2J3 (CYP2J3)	U39943	4
CYP3A1	X62086	4
Antioxidant enzymes		
Cu/Zu SOD	M25157	56
Other		
NADH–cytochrome b5 reductase	D00636	48
Glutathione synthetase	L38615	5
Glutathione reductase	U73174	5

were found to be increased in the aged liver (NADH–cytochrome b5 reductase and Cu/Zn SOD) were analyzed using rat-specific primers that yield PCR products of 660 and 540 bases, respectively; primers for rat GAPDH were added to the same reaction vessels as a constitutively expressed control. In addition, a control reaction using all of the PCR components, with the exception of reverse transcription cDNA, was performed to ensure that no contamination existed. Similar to the microarray analysis, the expression of both genes was increased in aged (24-month-old) livers compared with young (2-month-old) livers; GAPDH was expressed at similar levels in both samples (see Fig. 2, *A*). The expression of two genes noted to be increased in the aged human liver was further assessed. Analysis by RT-PCR demonstrated that both cytochrome P-450 1A1 (2,3,7,8 tetracholordibenzo-p-dioxin [TCDD]-inducible) and cytochrome P-450 2C18 (CYP2C18; clone 6b) were increased in the aged livers compared with the 1-year-old livers (see Fig. 2, *B*). GAPDH was expressed at similar levels in the samples.

To further confirm changes noted by our initial microarray analysis, we analyzed three additional young (2-month-old) and aged (24-month-old) rat livers for expression of NADH–cytochrome b5 reductase and Cu/Zn SOD (Fig. 3). Although the expression varied in the in vivo samples, increased expression (~10-fold) of NADH–cytochrome b5 reductase was noted in the three aged livers compared with the livers from the young rats (see Fig. 3, *A*). An analysis of the expanded group of rat liver samples demonstrated that the results for Cu/Zn SOD are less consistent with no significant change in expression noted in the aged and young livers (see Fig. 3, *B*). These results demonstrate the variability in the expression patterns in vivo and further emphasize the necessity of confirming the data obtained by microarray analyses using more conventional techniques (e.g., RT-PCR) and using samples from different animals.

Table 2. Subsets of genes that are increased in the aged human liver

Human genes	Gene bank	Fold increase
UDP-glucuronosyltransferase		
C19 steroid specific	U59209	3
Glutathione S–transferase		
Ha subunit 2 (GST)	M16594	19
Theta 2 (GSTT2)	L38503	8
Cytochrome P-450 enzymes		
P-450 1A1 (CYP1A1-TCDD-inducible)	K03191	9
CYP1A2	M31667	5
P-450 2C18 (CYP2C18)	M61853	3

Assessment of GST Functional Activity in Young and Aged Livers

We next determined whether the increased expression of members of the GST gene family was associated with increased functional activity. GST activity was assessed in the young and aged rat and human livers by testing the ability of these proteins to conjugate 1-chloro-2,4 dinitrobenzene (Fig. 4). Although not significant, there is a trend toward decreased GST activity in the aged rat liver compared with the young rat liver. A similar trend in GST enzyme activity is noted in human liver samples from the 78-year-old compared with those of the 1-year-old.

Collectively these findings demonstrate alterations in the expression of a number of the antioxidant and detoxification genes in the liver with aging. At least in the case of GST, increased expression of this gene in the aged liver does not appear to correlate with an increase in enzyme activity.

DISCUSSION

Aging is associated with changes in physiologic function and gene expression, which may greatly af-

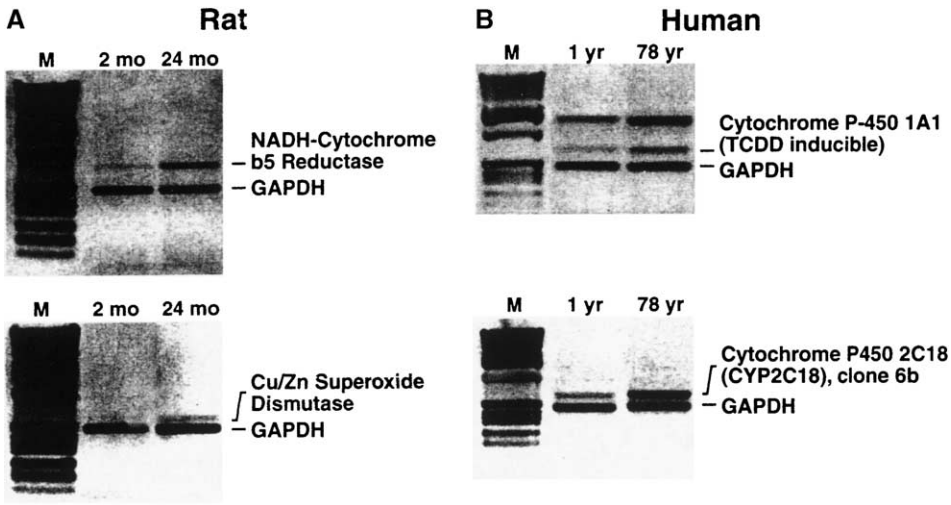


Fig. 2. RT-PCR analysis of rat and human expression patterns. **A,** To confirm rat gene expression increases, RNA extracted from young (2-month-old) and aged (24-month-old) rat livers was analyzed by a semiquantitative RT-PCR with primers for NADH–cytochrome b5 reductase and Cu/Zn SOD. **B,** RNA extracted from young (1-year-old) and aged (78-year-old) human livers was analyzed by a semiquantitative RT-PCR with primers for cytochrome P-450 (1A1) and cytochrome P-450 (2C18). The integrity of the RT reaction was confirmed by amplification using primers to the constitutively expressed GAPDH gene. Control experiments consisted of parallel reactions with all of the RT-PCR components except for the reverse transcription cDNA. M = molecular weight marker.

fect the functional responses of elderly patients after trauma or major operative procedures.[24] In our present study, we have used gene array techniques to analyze changes in the expression patterns of genes in the liver associated with aging. Increased expression of redox and detoxification enzymes, including the GST, UDP-glucuronosyltransferase, and cytochrome P-450 enzyme families, were noted in both aged rat and aged human liver samples. Other genes displaying increased expression in the rat liver included the antioxidant enzymes Cu/Zn SOD and NADH–cytochrome b5 reductase. These increased expression patterns were further corroborated by RT-PCR analysis of selected genes. Our findings demonstrate changes in the expression patterns of similar gene families in aged rat and aged human livers and suggest that gene array techniques will be useful to better delineate the changes in gene patterns associated with senescence.

The development of new technologies arising from the human genome project, such as DNA microarray analysis, will have an enormous impact on the future studies of normal cellular processes as well as disease states. The array-based methods, which involve the mobilization of thousands of cDNAs on a solid matrix, have the advantage of a high-output, direct, and rapid readout of hybridization results and immediate information on the expression patterns of numerous genes. Recent studies have used microarray techniques to analyze changes in gene expression patterns with aging. Lee et al.[25] analyzed skeletal muscle from mice of different ages and noted that 113 of 6347 genes displayed a greater than twofold change in expression levels as a function of age. Stress-related genes were noted to be increased, whereas genes involved in energy metabolism, biosynthetic pathways, and protein turnover were decreased with aging. In another study by Ly et al.,[26] gene expression patterns were measured in actively dividing fibroblasts isolated from young, middle-aged, and elderly humans and humans with progeria, a rare genetic disorder characterized by accelerated aging. Except for some common changes in expression patterns of the stress-responsive genes, there was little overlap between the alterations noted in these two studies,[25,26] suggesting that specific tissues may respond to aging differently. Taken together, our present study, as well as those of others,[26–30] confirm the fact that aging is associated with changes (either increases or decreases) in gene expression patterns. These changes are highly dependent on tissue type and species.

The accumulation over time of ROS, which are byproducts of metabolism and produced in all aerobic organisms, is thought to play a role in the pathogenesis of certain diseases and contribute to the changes noted with aging.[7,8,31,32] Antioxidants are "free-radical scavengers" that detoxify these potentially toxic byproducts;[6] alterations in the activity of

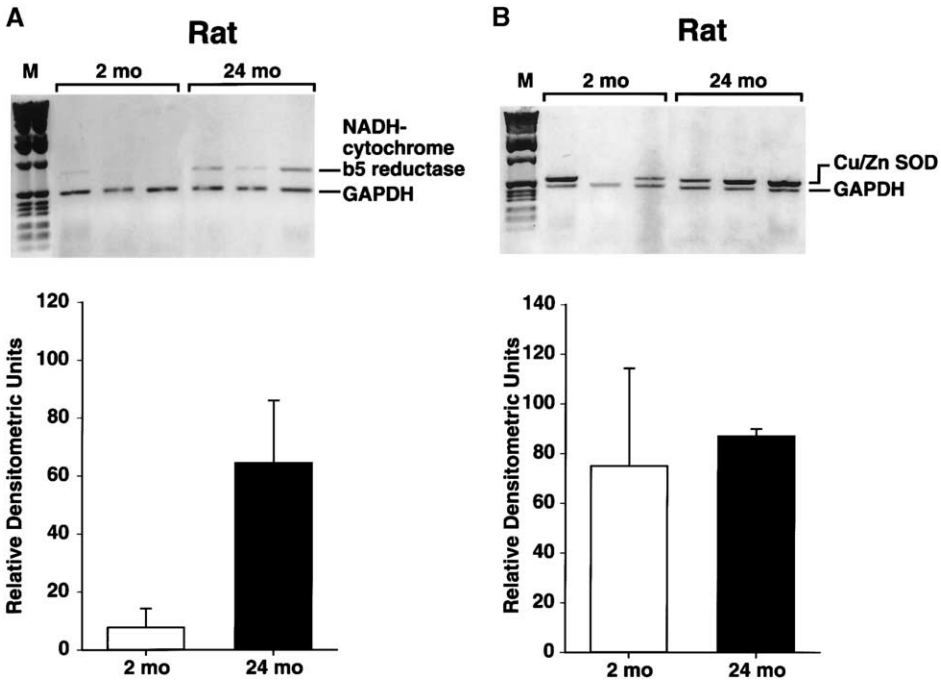


Fig. 3. RT-PCR analysis of additional in vivo young and aged rat livers. **A,** RNA was extracted from three additional young (2-month-old) and aged (24-month-old) rat livers. Samples were analyzed by RT-PCR with primers for NADH–cytochrome b5 reductase (**A**) and Cu/Zn SOD (**B**). Primers for the constitutively expressed GAPDH gene were run in the same reactions. RT-PCR results were quantitated using scanning densitometry and expressed as relative densitometric units after normalizing for GAPDH (mean ± SEM).

various antioxidants may allow for ROS accumulation. In our present study we have focused on changes that occur in the redox gene families in the liver. We found increased expression of several antioxidant enzymes including the members of the GST family, Cu/Zn SOD, and the membrane antioxidant NADH–cytochrome b5 reductase. To correlate expression patterns in the rat with changes in the human, we have assessed liver samples from an infant and an elderly patient and found, similar to that which occurs in the rat, an increase in the expression of members of the GST family. Although a powerful method to rapidly and simultaneously assess the expression changes of a multitude of genes, this technique represents a "snapshot" depicting changes in gene expression in a single tissue sample at a single time point. A number of factors may influence the expression patterns noted in an array analysis. Environmental factors including differences in diet, stress, and manner of tissue collection may alter the expression patterns detected by a single array. Therefore it is imperative that the changes noted by gene arrays be confirmed by more conventional techniques and, if possible, in tissues examined from multiple subjects. We have used RT-PCR to confirm changes noted in selected genes in both rat and human livers. Moreover, we have analyzed additional rat livers by RT-PCR to confirm changes in different animals of the same age. Of note, increased expression of NADH–cytochrome b5 reductase was consistently noted in aged livers compared to the young samples. In contrast, although the expression of Cu/Zn SOD was increased in the aged rat liver by gene array analysis, there was no difference in the expression pattern when other liver tissues were analyzed by RT-PCR. These studies further emphasize the importance of confirming the changes noted on gene arrays by other more conventional methods and also the need to analyze different tissue samples given the inherent variability found in individual in vivo specimens.

To further analyze, on a functional level, age-associated changes in GST, we measured GST enzyme activity in rat and human livers. Despite an apparent increase in gene expression in the aged liver, GST enzyme activity was not significantly altered in rat and human livers, although there was a trend toward decreasing GST activity in the aged livers compared with the young samples. Other studies have demonstrated a significant decrease in antioxidant enzyme activity with aging.[29,33,34] For example, both Rao et al.[29] and Semsei et al.[33] have shown a decrease in the antioxidant enzymes Cu/Zn SOD and catalase in rat livers with aging. However, Carrillo et al.[34]

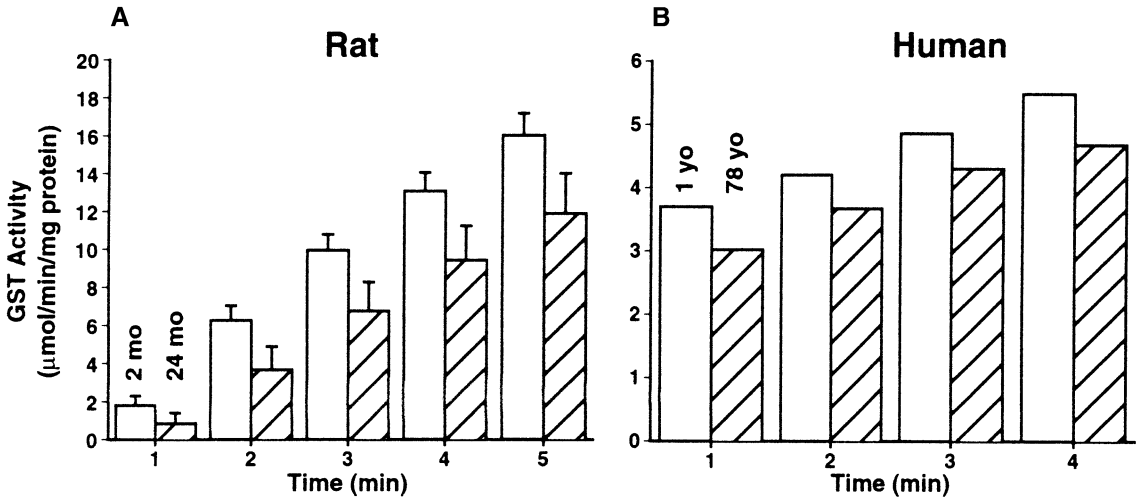


Fig. 4. Glutathione S–transferase activity. **A,** Cytosolic protein fractions from four young (2-month-old) (*open bars*) and four aged (24-month-old) (*single-hatched bars*) rat livers were analyzed by spectrometry for the ability of glutathione S–transferase *(GST)* to conjugate 1-chloro-2, 4-dinitrobenzene (CDNB) activity. Results are expressed in μmol/min/mg of protein sample (mean ± SEM). **B,** Cytosolic protein fractions from young (1-year-old) (*open bars*) and aged (78-year-old) (*single-hatched bars*) human samples analyzed by spectrophotometry for GST activity (μmol/min/mg protein sample).

found that, despite a decrease in catalase activity in aged male rats, catalase activity in aged female rats was increased compared with that in young rats. Similar differences in catalase activity have been described by Rikans et al.[35]

In humans the activities of the antioxidant enzymes Cu/Zn SOD, glutathione peroxidase, glutathione reductase, and GST were measured in individuals from 1 month to 63 years of age. An inverse correlation between age and the enzyme activities of Cu/Zn SOD, glutathione reductase, and GST was noted; however, in contrast, glutathione peroxidase activity increased with aging.[36] These findings suggest that there is a variable change in the antioxidant family members with aging. That is, a general decline in the activities of the antioxidants is not a universal phenomenon of aging and appears to be dependent on the particular enzyme analyzed. Gene array studies will further assist in characterizing the changes in a number of different gene families, which will then need to be assessed at the functional level. In our current study we have looked at two extremes of aging. It will be important to analyze intermediate-age groups in the future to determine whether a gradient of expression changes occur with aging or, conversely, if a certain age must be achieved for these alterations in expression to occur. In addition, an important question to address in the future is the effect of stress in the elderly on the activity of these enzymes.

Members of the cytochrome P-450 family of genes were noted to be increased in the aged rat and human livers. These enzymes serve to detoxify lipophilic compounds into water-soluble metabolites by oxygenation reactions. In addition, other functions of members of this family include hydroxylation of steroids and biotransformation of drugs.[37] Although our findings suggest an increase in the expression levels of these genes, we have not assessed the functional activity of these enzymes with aging to determine whether the enzyme activity correlates with actual gene expression levels. This is another important aspect of gene array studies that must be considered. That is, gene expression changes may not correlate with protein expression or functional activity in the cell. Therefore the significance of expression changes noted by gene array analysis should be interpreted with caution with regard to their actual consequences in the cell.

CONCLUSION

Our results, obtained by gene array analysis, provide insights into the changes in gene expression patterns associated with the aging process. Further, our results demonstrate similar increases in the expression of antioxidant genes, such as GST and NADH–cytochrome b5 reductase, in the livers of aged rats and humans. The expression of members of the cytochrome P-450 family of genes was also increased both in rats and humans, suggesting common regulatory pathways for age-associated changes in the

mammalian liver. Delineating the gene expression profiles that are altered with aging will provide a better understanding of the functional changes that occur in various organs with senescence. This improved understanding may provide novel approaches to the treatment of aged patients.

We thank Maria Cristina Carillo, Ph.D. (Universidad Nacional de Rosario, Rosario, Argentina), for her help with the GST assay methodology. We also thank Mr. Tatsuo Uchida for statistical analysis, and Eileen Figueroa and Karen Martin for manuscript preparation.

REFERENCES

1. Day JC. Population projections of the United States by age, sex, race and Hispanic origin: 1993–2050. In Current Population Reports. Washington, D.C.:U.S. Department of Commerce Bureau of the Census, 1993, pp 25–1104.
2. Anonymous. CDC Advance Data No. 301. Atlanta, Centers for Disease Control and Prevention: August 31, 1998.
3. Rosenthal RA, Zenilman ME. Surgery in the elderly. In Townsend CM Jr, Beauchamp RD, Evers BM, Mattox KL, eds. Sabiston Textbook of Surgery, 16th ed. Philadelphia:WB Saunders, 2001, pp 226–246.
4. Powers JH. Surgery of the Aged and Debilitated. Philadelphia: WB Saunders, 1968.
5. Zenilman ME. Surgery in the elderly. Curr Probl Surg 1998;35:99–179.
6. Knight JA. The biochemistry of aging. Adv Clin Chem 2000;35:1–62.
7. Shigenaga MK, Hagen TM, Ames BN. Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci U S A 1994;91:10771–10778.
8. Harman D. Aging: A theory based on free radical and radiation chemistry. J Gerontol 1956;11:298–300.
9. Yu BP. Cellular defenses against damage from reactive oxygen species. Physiol Rev 1994;74:139–162.
10. Kristal BS, Yu BP. An emerging hypothesis: Synergistic induction of aging by free radicals and Maillard reactions. J Gerontol 1992;47:B107–B114.
11. McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969;244:6049–6055.
12. Villalba JM, Navas P. Plasma membrane redox system in the control of stress-induced apoptosis. Antioxid Redox Signal 2000;2:213–230.
13. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci U S A 1993;90:7915–7922.
14. Amicarelli F, Di Ilio C, Masciocco L, Bonfigli A, Zarivi O, D'Andrea MR, Di Giulio C, Miranda M. Aging and detoxifying enzymes responses to hypoxic or hyperoxic treatment. Mech Ageing Dev 1997;97:215–226.
15. Grant DM. Detoxification pathways in the liver. J Inherit Metab Dis 1991;14:421–430.
16. Gonzales FJ. The molecular biology of cytochrome P-450. Pharmacol Rev 1989;40:243–288.
17. Evers BM, Townsend CM Jr, Thompson JC. Organ physiology of aging. Surg Clin North Am 1994;74:23–39.
18. Chesky JA, Rockstein M. Life span characteristics in the male Fischer rat. Exp Aging Res 1976;2:399–407.
19. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–159.
20. Ehlers RA, Kim S, Zhang Y, Ethridge RT, Murrilo C, Hellmich MR, Evans DB, Townsend CM Jr, Evers BM. Gut peptide receptor expression in human pancreatic cancers. Ann Surg 2000;231:838–848.
21. Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 1974;249:7130–7139.
22. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.
23. Carrillo MC, Favre C, Carnovale CE, Monti JA, Alvarez ML, Scapini C, Pisani GB, Lugano MC. Involvement of mu class glutathione S-transferase subunit M2 (rGST M2) levels in the initiation and promotion of hepatocellular carcinogenesis in old rats. Exp Gerontol 2001;36:255–265.
24. Bengton VL, Schaie KW. Handbook of Theories of Aging. New York: Springer, 1999.
25. Lee CK, Klopp RG, Weindruch R, Prolla TA. Gene expression profile of aging and its retardation by caloric restriction. Science 1999;285:1390–1393.
26. Ly DH, Lockhart DJ, Lerner RA, Schultz PG. Mitotic misregulation and human aging. Science 2000;287:2486–2492.
27. Orr WC, Sohal RS. Extension of life-span by overexpression of superoxide dismutase and catalase in Drosophila melanogaster. Science 1994;263:1128–1130.
28. Larsen PL. Aging and resistance to oxidative damage in Caenorhabditis elegans. Proc Natl Acad Sci U S A 1993;90:8905–8909.
29. Rao G, Xia E, Richardson A. Effect of age on the expression of antioxidant enzymes in male Fischer F344 rats. Mech Ageing Dev 1990;53:49–60.
30. Han E, Hilsenbeck SG, Richardson A, Nelson JF. cDNA expression arrays reveal incomplete reversal of age-related changes in gene expression by calorie restriction. Mech Ageing Dev 2000;115:157–174.
31. Kensler TW, Taffe BG. Free radicals in tumor promotion. Adv Free Radic Biol Med 1986;2:347–387.
32. Oberley LW. Free radicals and diabetes. Free Radic Biol Med 1988;5:113–124.
33. Semsei I, Rao G, Richardson A. Changes in the expression of superoxide dismutase and catalase as a function of age and dietary restriction. Biochem Biophys Res Commun 1989;164:620–625.
34. Carillo MC, Kanai S, Sato Y, Kitani K. Age-related changes in antioxidant enzyme activities are region and organ selective as well as sex in the rat. Mech Ageing Dev 1992;65:187–198.
35. Rikans LE, Moore DR, Snowden CD. Sex-dependent differences in the effects of aging on antioxidant defense mechanisms of rat liver. Biochim Biophys Acta 1991;1074:195–200.
36. Ceballos-Picot I, Trivier JM, Nicole A, Sinet PM, Thevenin M. Age-correlated modifications of copper-zinc superoxide dismutase and glutathione-related enzyme activities in human erythrocytes. Clin Chem 1992;38:66–70.
37. Imaoka S, Fujita S, Funae Y. Age-dependent expression of cytochrome P-450s in rat liver. Biochim Biophys Acta 1991;1097:187–192.

Discussion

Dr. S. Strasberg (St. Louis, MO): I am really not qualified to ask a question about this study except in terms of experimental design. I find the age selection that you used to be somewhat unusual and, as a result, I find it difficult to see how you arrived at your conclusions. You have chosen to study two persons: one, a very young human who is in development, and the other, a very old human. If you wanted to make the point that genes get turned on with aging, it would seem to me that you need persons of other ages. I think a young adult (i.e., a fully mature human) should be used for comparison to very old patients rather than an infant if one is to conclude that genes are turned off with aging. This study could likewise be interpreted to show that certain genes are turned off during development.

Dr. R. Thomas: That is a very good point. We would like to expand our studies and perform more gene array experiments using rat and human samples of different ages. We chose to use our array data as a "snapshot" of expression changes; therefore our samples were of young and aged subjects. I think the point is valid that this is not a comprehensive look at the total expression changes throughout development of either rats or human.

Dr. M. Sarr (Rochester, MN): First of all, congratulations on using RT-PCR to confirm the chip analysis; however, your N value here is only 1 for the humans, and I am sure you have been expecting this question. You are making fairly broad generalizations based on one liver in a child and one liver in an adult. That is potentially dangerous.

Dr. Thomas: I agree. Again, we understand that this is a limited number of samples. We plan to extend these findings. This experiment was a starting point.

Dr. J. Cullen (Iowa City, IA): A number of the genes that you are looking at or the antioxidants that you are looking at are actually induced or increased because of an increase in ROS. In aging there is an increase in ROS because there is actually a decrease in the major antioxidant enzymes, manganese SOD, copper zinc SOD, catalase and glutathione peroxidase. Have you looked at the changes either in the immunoreactive protein or the activity of those major antioxidant enzymes?

Dr. Thomas: We did not look specifically at the enzyme function of manganese SOD, Cu/ZN SOD, catalase, or glutathione peroxidase. Our gene array demonstrated increases in Cu/ZN SOD; however, subsequent RT-PCR studies identified variable results.

Furthermore, your point is well taken. Although we confirmed our expression changes by RT-PCR, work is still needed to complete these investigations at the protein and enzyme function level.

Dr. S. Ashley (Boston, MA): This is a nice study. I might have been more interested in what got turned off with age and what protective mechanisms were lost with UCVC. Was there anything that was consistent?

Dr. Thomas: We have not completely investigated the genes that demonstrated a decrease with aging. The decision to investigate genes that increase with aging was made because a general conception of the aging process is that there is a general decline in gene expression and physiologic function.

Invited Discussion—Expert Commentator

Henry A. Pitt (Milwaukee, WI): The paper by Dr. Thomas et al. from Galveston explored the effects of aging on gene expression patterns in the liver. As usual, Dr. Evers and his colleagues have brought the latest research techniques to a surgical meeting. This gene chip technology is a very powerful tool for identifying differences between groups or, in this case, over time, a long time, with aging.

One of the disadvantages, however, is that each study provides more data than can be easily assimilated. As a result, years of follow-up work are required after a gene chip analysis. Another concern about a study like this one we hear today is that large amounts of data are gathered from very few tissue specimens. Therefore, the question arises as to the generalizability of the information.

Another issue is whether data from the liver apply to other organs. In addition, although data are available from this study in over 9,000 rat and 13,000 human genes, this sample is only a small percentage of all the rat and human genome. Therefore, the choice of genes on a chip does limit the conclusions that can be drawn from an individual analysis, even with years of follow-up.

Mechanisms of Tolerance Induction After Rat Liver Transplantation: Intrahepatic CD4$^+$ T Cells Produce Different Cytokines During Rejection and Tolerance in Response to Stimulation

Christoph Otto, Ph.D., Jens Kauczok, M.D., Natascha Martens, Ulrich Steger, M.D., Ingo Möller, M.D., Detlef Meyer, M.D., Wolfgang Timmermann, M.D., Karin Ulrichs, Ph.D., Heinz-Jochen Gassel, M.D.

Increasing evidence supports the existence of regulatory T cells that may inhibit the allogeneic immune response after transplantation by secreting regulatory cytokines. To determine whether rat liver tolerance is associated with intrahepatic regulatory T cells secreting a characteristic cytokine profile, we analyzed the cytokine production of freshly isolated intragraft CD4$^+$ T cells at different times postoperatively by semiquantitative reverse transcription–polymerase chain reaction and by enzyme-linked immunosorbent assay before and after in vitro stimulation. Orthotopic arterialized liver transplantation was performed in two allogeneic rat strain combinations, one with fatal acute rejection (DA-to-LEW) and one with long-lasting tolerance (LEW-to-DA) without immunosuppression despite a complete major histocompatibility complex mismatch (spontaneous liver tolerance). Liver allografts of both groups showed continuously increasing cellular infiltration between day 3 and day 7 after transplantation. In this inflammatory situation, very low levels of interleukin-13 were detectable directly after cell isolation, as well as after in vitro stimulation. However, after 30 days, intrahepatic CD4$^+$T cells in the tolerance group were then able to express elevated messenger RNA levels of the anti-inflammatory cytokine interleukin-13 in response to stimulation. This result indicates the presence of an intragraft Th2-like CD4$^+$ T cell population, which may have a regulatory function in the induction of liver tolerance. (J GASTROINTEST SURG 2002;6:455–463.)

KEY WORDS: Tolerance, transplantation, cytokines, CD4$^+$ T cells, interleukin-13 mRNA

The liver allograft appears to be an immunologically "privileged" organ that facilitates the induction of donor-specific tolerance after transplantation. This is a very important characteristic because the special situation after transplantation normally requires life-long suppression of the recipient's immune system to inhibit an immune response against the major histocompatibility complex (MHC)–incompatible organ. This life-long immunosuppression entails serious complications such as drug-specific side effects; bacterial, fungal, and viral infections; and an increased incidence of malignancies.[1]

Therefore a major goal in transplantation research is the induction of donor-specific tolerance in an adult immune system. Understanding of the cellular and molecular mechanisms of liver-induced tolerance is crucial for the success of clinical transplantation. Recent data indicate that immunoregulatory CD4$^+$ T cells play an important role in preventing allograft rejection.[2,3] The mechanism underlying the ability of CD4$^+$ T cells to prevent allograft rejection is not known in detail; however, it seems that they secrete cytokines in a special profile.[4,5] It is well established that rat CD4$^+$ T cells can be phenotypi-

Presented in part at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (poster presentation).

From the Experimental Transplantation Immunology Unit of the Department of Surgery (J.K., N.M., U.S., I.M., K.U.) and the Department of Surgery (H.-J.G., D.M., W.T.), University of Würzburg, Würzburg, Germany.

Supported by the Federal Ministry of Education and by research funds supplied to the Interdisciplinary Centre for Clinical Research (IZKF) of the University of Würzburg (research project grant number 01 KS 9603).

Reprint requests: Christoph Otto, Ph.D., Department of Surgery (Director: Prof. Dr. med. A. Thiede), Experimental Transplantation Immunology Unit of the Department of Surgery, University of Würzburg, Josef-Schneider-Str. 2, D-97080 Würzburg, Germany.
e-mail: chotto@chirurgie.uni-wuerzburg.de

1091-255X/02/$—see front matter
PII: S1091-255X(02)00012-4

cally divided into subsets based on their expression of the leukocyte common antigen CD45.[6] The two $CD4^+$ subpopulations, $CD45RC^+$ and $CD45RC^-$ T cells, have different cytokine profiles: $CD45RC^+$ $CD4^+$ T cells produce interleukin (IL)-2 and interferon(IFN)-γ, but express only small amounts of IL-4 mRNA, whereas $CD45RC^-$ $CD4^+$ T cells produce less IL-2 and IFN-γ but express high amounts of IL-4 mRNA.[7] Important for the situation after transplantation is that $CD45RC^+$ $CD4^+$ T cells are most effective in inducing allograft rejection, whereas $CD45RC^-$ $CD4^+$ T cells are not.[8]

The rat liver allograft model is unique because in some fully allogeneic rat strain combinations, the liver is accepted spontaneously. Liver allografts from Lewis (LEW) donors, for example, are spontaneously tolerated in dark agouti (DA) recipients (LEW-to-DA combination, tolerance [TOL] group), whereas in the reciprocal DA-to-LEW combination, the liver allograft will be rejected (rejection [REJ] group) if no immunosuppression is used.

The immunomodulatory effect of intrahepatic T lymphocytes was demonstrated by Sun et al.[9] in a rat model of spontaneous liver allograft acceptance. In the present study, we examined the influence of the allogeneic situation on the balance of both $CD4^+$ T cell subsets, $CD45RC^+$ and $CD45RC^-$, in liver grafts after transplantation. To explain the possible immunoregulatory role of a special subpopulation of intrahepatic $CD4^+$ T cells in liver tolerance, we examined the cytokine production of purified intrahepatic $CD4^+$ T cells at different time points after transplantation in the phase of immune reactivity and in the phase of acceptance from the TOL and the REJ groups. The balance between $CD45RC^+$ and $CD45RC^-$ $CD4^+$ T cells was analyzed by flow cytometry, and the cytokine profile of these cells was compared to those of intrahepatic $CD4^+$ T cells isolated from syngeneic (SYN; control) liver grafts. The aim of this study was to clarify whether possible regulatory $CD4^+$ T cells could be detected in accepted liver allografts by a shift of cytokine production from a Th1- to a Th2-related cytokine profile.

MATERIAL AND METHODS

Orthotopic Rat Liver Transplantation and Experimental Design

Six- to 8-week-old male inbred DA rats ($RT1^{avl}$), weighing 180 to 200 g, served as recipients of LEW rat ($RT1^l$) livers. All animals were purchased from Charles River Laboratories (Sulzfeld, Germany) and were housed in accordance with national and institutional animal care policies. Orthotopic rat liver transplantation was performed in an arterialized model with hepatic artery revascularization, as originally described by Lee in 1975 and modified by us.[10] The death of one animal before day 2 was attributed to technical errors, and it was excluded from the study. Rats undergoing orthotopic liver transplantation were divided into the following three groups: SYN (LEW-to-LEW; n = 30); REJ (DA-to-LEW; n = 28); and TOL (LEW-to-DA; n = 48). To assess intrahepatic $CD4^+$ T cells within liver grafts, three animals each from the SYN and TOL groups were killed in the early phase on days 3, 5, 7, and 14 and in the late phase on days 30, 60, and 100 after transplantation. From the REJ group, intrahepatic $CD4^+$ T cells were analyzed on days 3, 5, and 7 after transplantation (Tables 1 and 2).

Evidence of Donor-Specific Tolerance

To assess the development of tolerance in the recipients of the TOL group, long-term survivors (>100 days) received either donor-specific (LEW)

Table 1. Number of rats undergoing liver transplatation needed for each time point (from day 3 to day 100)*

Group	Strain combination	Postoperative days of leukocyte isolation							n†
		3	5	7	14	30	60	100	
REJ	DA-LEW	8‡	6	8§	—	—	—	—	22
TOL	LEW-DA	6	6	6	6	6	6	6	42
SYN	LEW-LEW	6	ND	ND	6	ND	6	6	24

ND = Analysis not done at this time point.
*The intrahepatic leukocytes from two livers were pooled to perform flow cytometric analysis and $CD4^+$ T-cell purification.
†Total number of transplanted animals in each experimental group.
‡Number of transplanted animals for each time point; intrahepatic leukocytes from two animals were pooled to isolate intrahepatic $CD4^+$ T cells needed for cytokine detection by ELISA and cytokine mRNA by RT-PCR as described in Material and Methods.
§Day 7 was the last time point for analysis of intrahepatic $CD4^+$ T cells from the REJ group because the condition of these animals deteriorated dramatically.

Table 2. Number of rats undergoing liver tranplantation used for determination of recipient survival times

Group	Strain combination	Survival (days)	n
REJ	DA-LEW	9, 10 (×4), 12	6
TOL	LEW-DA	>100*	6†
SYN	LEW-LEW	>100*	6

*All animals in the SYN group and the three animals in the TOL group receiving heterotopic LEW hearts were killed without any signs of rejection 200 days after liver transplantation.
†For determination of tolerance, DA rats undergoing liver transplantation received a heterotopic cardiac allograft from either LEW (n = 3) or Wister-Furth (n = 3) rats.

or third-party (Wistar-Furth) hearts (n = 3 each). Heterotopic cardiac transplantation was performed according to the method of Ono and Lindsey.[11] Graft rejection was considered as the complete cessation of palpable cardiac contractions and was confirmed histologically.

Isolation of Intrahepatic Leukocytes

Rats were anesthetized with isoflurane. After laparotomy, the portal vein was exposed and cannulated with a 21-gauge needle. The liver was perfused with 10 ml of 4° C saline solution through the portal vein after the inferior vena cava was opened above the liver. The blanched liver was then excised, diced into small pieces with scissors, and gently pressed through a fine steel sieve. This cell suspension was incubated for 30 minutes at 37° C in a shaking water bath at 80 rpm in the presence of 100 U/ml (final concentration) collagenase type IV (Sigma, Deisenhofen, Germany) and 0.001% (final concentration) DNase I (Roche Diagnostics GmbH, Mannheim, Germany). Hepatocyte pellets were obtained from the cell suspension by centrifugation at 16 *g* for 2 minutes at 16° C. The leukocyte-enriched supernates were pooled, centrifuged, and resuspended in 15 ml of sterile 36% (v/v) Percoll solution (Amersham Pharmacia Biotech, Freiburg, Germany). This solution was layered carefully over 15 ml of 72% (v/v) Percoll solution in a 50 ml polypropylene centrifuge tube. The gradient was centrifuged at 20° C for 30 minutes at 1118 *g*. Hepatocytes and cell debris on the top of the 36% (v/v) Percoll solution were discarded, and intrahepatic leukocytes at the interphase were recovered and washed two times with phosphate-buffered saline solution (PBS; 140 mmol/L NaCl, 2.7 mmol/L KCl, 7.2 mmol/L $Na_2HPO_4 \times 2H_2O$, 1.47 mmol/L KH_2PO_4, pH 7.2). The total number of PBS-washed viable cells (excluding trypan blue) was determined using a hemocytometer. For further applications, the intrahepatic leukocytes were adjusted to 1×10^7 cells/ml.

Flow Cytometry

Flow cytometric analysis was performed with a Becton Dickinson FACScan flow cytometer (Beckton Dickinson, Franklin Lakes, New Jersey) with an argon ion laser at 488 nm for excitation. List mode files representing 10,000 cells were collected for each sample. Data files were analyzed with the WinMDI program (The Scripps Research Institute, La Jolla, California). Data were gated according to the forward and side scatter signals of the intrahepatic leukocyte population. Per analysis 5×10^5 cells were stained in 50 μl PBS for 20 minutes at 4° C. The monoclonal antibodies W3/25 (anti-rat CD4) [12] and OX-22 (anti-rat CD45RC)[13] were used in combination for typing the $CD45RC^+$ and $CD45RC^-CD4^+$ T-cell subpopulations. All monoclonal antibodies were purchased from Serotec (Oxford, United Kingdom).

Immunomagnetic Separation of Intrahepatic $CD4^+$T Cells

$CD4^+$ T cells were isolated from intrahepatic leukocytes in two steps: in the first step all OX-8 (CD8α-chain)–positive cells ($CD8^+$ and natural killer cells) were depleted in order to subsequently select the remaining $CD4^+$ lymphocytes using the monoclonal antibody R7.3 (anti-αβ TCR). The leukocytes were incubated with monoclonal antibody OX-8 (0.3 μg monoclonal antibody for 10^6 positive cells) for 30 minutes at 4° C. Dynabeads (CELLectionTM Pan Mouse IgG Kit) (Dynal GmbH, Hamburg, Germany) were added at a ratio of four beads per target cell. Positive selection with monoclonal antibody R7.3 was performed under the same conditions as described for monoclonal antibody OX-8. In the second step, the beads were removed from the positively selected $CD4^+$ T lymphocytes by gently resuspending the cells in 200 μl prewarmed (37° C) RPMI 1640 culture medium containing 166 μg DNase I (Roche Diagnostics GmbH, Mannheim, Germany). The RPMI 1640 medium was supplemented with 10% heat-inactivated fetal calf serum, 5 mmol/L HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, 2×10^{-5} mol/L 2-mercaptoethanol, 1 mmol/L sodium pyruvate, and 1% nonessential amino acids (all reagents from Life Technologies GmbH, Karlsruhe, Germany). After 20 minutes, the tube was placed in a magnet (Dynal MPC) for 1 minute and the supernate with the released cells was removed to a second tube containing 200 μl RPMI

medium. The positively selected $CD4^+$ T cells were counted and the purity (>95%) was determined by flow cytometry.

In Vitro Stimulation

Freshly isolated intrahepatic $CD4^+$ T cells (1×10^5 cells per well of a 96-well flat-bottomed culture plate) were cultured in 200 μl complete RPMI 1640 medium with plate-bound goat anti-mouse monoclonal antibody (30 μg/ml) and incubated at 37° C in 6% CO_2 atmosphere for 3 hours. Before incubation, the monoclonal antibody R7.3 positively selected $CD4^+$ T cells were incubated with anti-CD28 (1 μg monoclonal antibody for 6×10^5 cells) for 30 minutes. For control purposes, positively selected $CD4^+$ T cells were not stimulated with monoclonal antibody JJ319 but were cultured under similar incubation conditions.

Quantification of IL-2 and IL-4 in Supernate

Cell-free supernate from six parallel experiments was collected to determine cytokine levels using enzyme-linked immunosorbent assay (ELISA) kits specific for rat IL-2 and IL-4 (Biosource International Inc., Camarillo, CA) in accordance with the recommendations of the manufacturer. The lower limits of detection were 5 pg/ml for IL-2 and 2 pg/ml for IL-4.

Cytokine Reverse Transcription–Polymerase Chain Reaction

Total RNA was prepared from 6×10^5 $CD4^+$ T cells treated with TRIzol Reagent (Life Technologies GmbH, Karlsruhe, Germany) according to the manufacturer's recommendations. The air-dried RNA was dissolved in 40 μl RNA storage solution (1 mmol/L sodium citrate, pH 6.4) (Ambion; AMS Biotechnology, Wiesbaden, Germany). For cDNA synthesis, 5 μl (nearly 0.2 μg) RNA was reverse transcribed with MuLV reverse transcriptase (RT; 2.5 U/μl final concentration) and Oligo $d(T)_{16}$ primer (2.5 μmol/L final concentration) using the Gene Amp RNA polymerase chain reaction (PCR) kit (Applied Biosystems GmbH, Weiterstadt, Germany). Five microliters from the resulting cDNA solution were then amplified using specific oligonucleotides (the forward primer was labeled with Digoxigenin [DIG]) for the rat cytokines IL-10 and IL-13 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a "housekeeping gene" (synthesized by MWG, Ebersberg, Germany) in a volume of 50 μl 1× PCR buffer (Applied Biosystems GmbH). The conditions (final concentrations) were 1.5 mmol/L $MgCl_2$, 0.1 mmol/L dNTPs, 0.25 μmol/L primer, and 0.05 U/μl AmpliTaq polymerase (Applied Biosystems GmbH). The primers were based on published sequences.[14–16] Annealing temperature, number of cycles, and the predicted product size for each primer were as follows: IL-10 = 55° C for 30 seconds, 28 cycles, 370 base pairs; IL-13 = 55° C for 30 seconds, 30 cycles, 280 base pairs; GAPDH = 62° C for 30 seconds, 20 cycles, 319 base pairs. Amplifications were performed under the following conditions: 95° C for 30 seconds and 72° C for 40 seconds. The final extension step was performed by one cycle at 72° C for 7 minutes.

ELISA-Based Quantification of PCR Products

The amount of PCR amplicons was quantified using an ELISA-based method as described.[17] Forty microliters of the PCR mixture were hybridized with 0.5 mmol/L of specific biotinylated oligonucleotides for 30 minutes at 55° C in the PCR cycler. Subsequently 5 μl of the DNA hybrids was diluted with 200 μl hybridization buffer (Roche Diagnostics GmbH, Mannheim, Germany) and transferred to Streptavidin-coated ELISA plates (Roche Diagnostics GmbH). After incubation with anti-DIG peroxidase-labeled Fab fragments (Roche Diagnostics GmbH) and then with peroxidase substrate solution (Roche Diagnostics GmbH), the reaction intensity was measured as optical density (OD) at 450 nm using an ELISA reader. Results were expressed using the following equation: ratio (cytokine/GAPDH) expression = (OD_{450} cytokine/OD_{450} GAPDH).

Statistical Analysis

Experimental data were evaluated for significant differences using the Mann-Whitney U test; differences of $P < 0.05$ were considered significant.

RESULTS

Liver allografts of the TOL group (LEW-to-DA) were accepted spontaneously without immunosuppression. Liver allografts from LEW donors survived long term (>100 day) in DA recipients without immunosuppression, despite a complete MHC mismatch (spontaneous liver tolerance group, n = 6). To assess the development of liver induced-tolerance, donor (LEW)-specific and third-party (Wistar-Furth)–specific cardiac allografts (n = 3 each) were transplanted heterotopically into long-term surviving (>100 days) DA recipients of LEW livers. Whereas LEW cardiac grafts were accepted for more than 100 days with no signs of rejection, the Wistar-Furth (third-party) cardiac grafts were re-

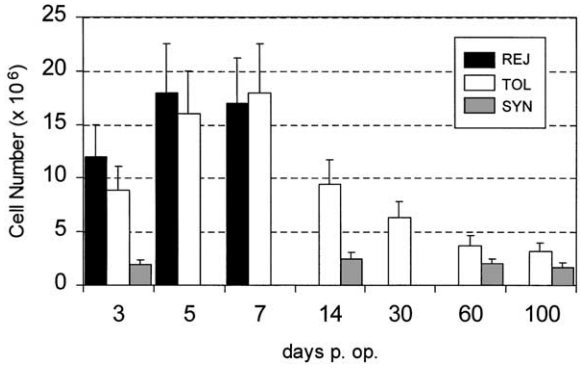


Fig. 1. The number of intrahepatic leukocytes from the rejection (*REJ*: DA-to-LEW), tolerance (*TOL*: LEW-to-DA), and syngeneic (*SYN*: LEW-to-LEW) groups was analyzed at different time points after transplantation. The cellular infiltrations were comparable in rejected and nonrejected liver allografts and were indicative of an inflammatory situation. For the REJ group, the last day of analysis was day 7 because the condition of these animals deteriorated dramatically after that. The mean and standard deviation of leukocyte values from four animals in each group are shown.

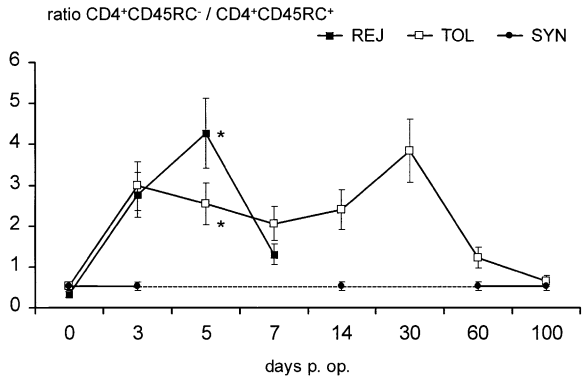


Fig. 2. Phenotyping of $CD4^+$ T-cell subpopulations with monoclonal antibodies W3/25 ($CD4^+$ T cells) and OX-22 (CD45RC). Shown are the $CD45RC^-/CD45RC^+$ ratios of intrahepatic $CD4^+$ T cells isolated from liver allografts (*REJ* and *TOL*), as well as from isografts (*SYN*). The increase in $CD45RC^-$ $CD4^+$ T cells correlates with an increase in activated (IL-2 receptor positive) T cells in the early phase. This observation underlines the fact that long-term survival in the TOL group is associated with an early immune activation. The data represent the $CD4^+$ $CD45RC^-/CD4^+$ $CD45RC^+$ ratio within the intrahepatic leukocyte population. Data are shown as mean ± standard deviation for four to six animals of each combination. *Difference between the REJ and TOL group is significant.

jected within 8 days. These hearts showed cellular infiltration with myocyte necrosis (not shown). In the REJ group (DA-to-LEW; n = 6), the DA liver allografts lost function within 12 days, and the recipients died of acute graft rejection as shown by histologic examination using hematoxylin and eosin stain. Recipients (n = 6) of the syngeneic control allografts (LEW-to-LEW) survived long term without any signs of allograft dysfunction.

Quantification of intrahepatic leukocytes revealed dynamic changes in cell number in both the REJ and TOL groups in the early phase after transplantation. The number of isolated intrahepatic leukocytes was determined per liver allograft using a hemocytometer. Liver allografts of both allogeneic combinations showed a progressive infiltration by leukocytes that continuously increased until day 7 (Fig. 1). The proportion of intrahepatic leukocytes was largest on day 7 and increased to $17 \pm 4.3 \times 10^6$ cells per gram of liver tissue compared to the SYN group with $1.9 \pm 1.3 \times 10^6$ cells per gram of liver. In the TOL group this infiltrate decreased continuously after day 14 to $3.2 \pm 0.13 \times 10^6$ cells per gram of liver on day 100. The number of leukocytes in the SYN group was $1.7 \pm 0.41 \times 10^6$ cells per gram liver on day 100. Histologic evaluation of liver biopsies supported these quantification results (not shown). The liver allografts of both allogeneic combinations, the REJ and the TOL groups, showed severe acute cellular rejection with prominent portal and lobular infiltration in the early phase (days 3, 5, and 7) after transplantation. This infiltrate resolved spontaneously in liver allografts in the TOL group in the late phase after transplantation (days 30, 60, and 100).

Persistence of Phenotype-Different $CD4^+$ T Cells in Both the REJ and TOL Groups

Beginning immediately after transplantation, the ratio of $CD45RC^-/CD45RC^+$ $CD4^+$ T cells increased over time in both allogeneic groups but was significantly greater in the REJ group (Fig. 2). This expansion of $CD45RC^-$ $CD4^+$ T cells goes parallel with increasing amounts of activated (IL-2 receptor positive) T cells (not shown). This indicates that the recipient's immune system had initiated an alloresponse against antigens transferred with the MHC-incompatible liver allograft. The quotient of $CD45RC^-/CD45RC^+$ declined after day 5, a reduction that was more dramatic in the REJ group than in the TOL group (see Fig. 2). Whereas the animals in the REJ group died within 12 days, animals in the TOL group survived this crisis. However, these animals showed a second increase in the $CD45RC^-/CD45RC^+$ quotient after day 14. After day 30, this quotient dropped continuously, reaching levels on day 100 comparable to those of the SYN group.

IL-2 production increased more in the REJ group than in the TOL group. The IL-2 production by intrahepatic $CD4^+$ T cells isolated from the REJ

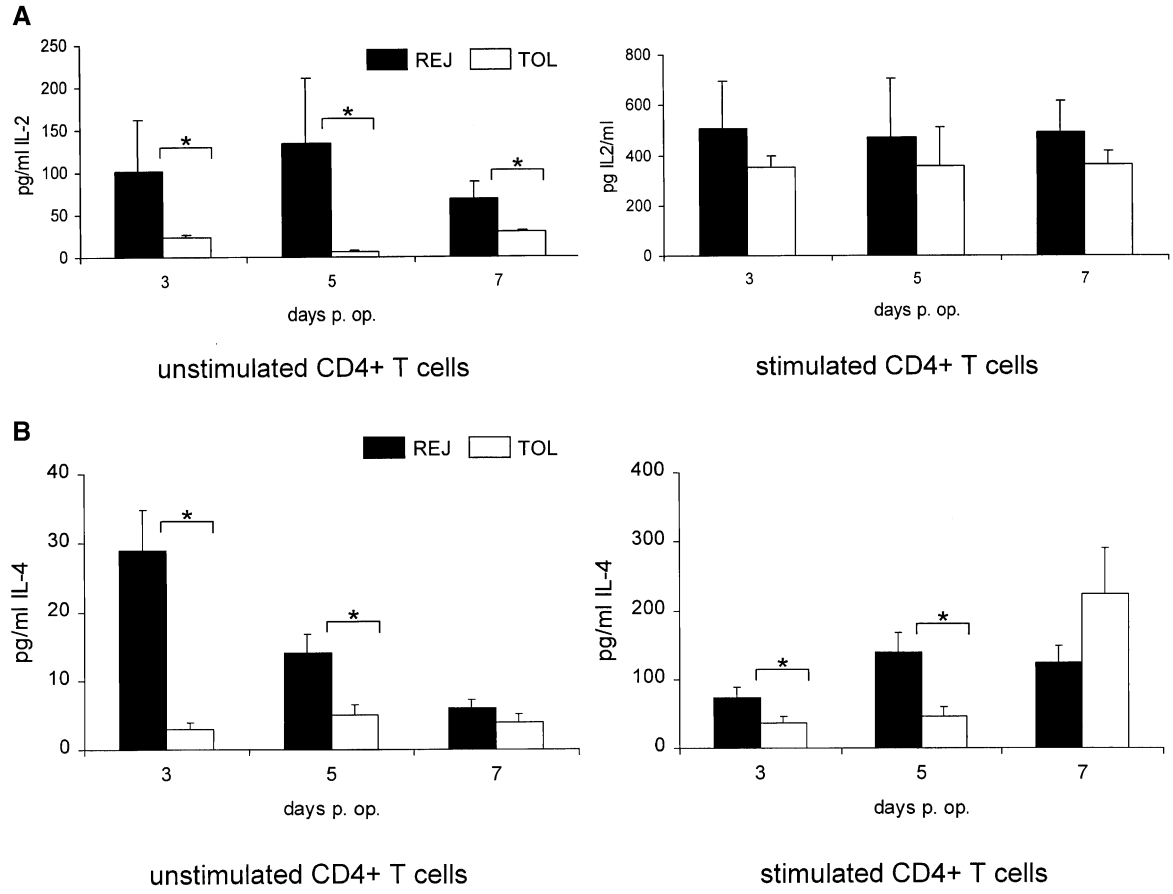


Fig. 3. IL-2 (**A**) and IL-4 (**B**) cytokine production by intrahepatic $CD4^+$ T cells isolated from liver allografts in the rejection (*REJ*) and tolerance (*TOL*) groups in the early (days 3, 5, and 7) phase after liver transplantation. The inflammatory situation in the early phase after transplantation is influenced by Th1 (IL-2) and Th2 (IL-4) cytokines. Shown are the mean and standard deviation of the cytokine values obtained from supernates harvested after 3 hours of culture under unstimulated conditions. The results shown are representative of four animals. *Significant.

group in the early phase after transplantation revealed a strong intrahepatic inflammatory milieu. These $CD4^+$ T cells secreted more IL-2 into the supernate than the $CD4^+$ T cells isolated from LEW livers of the TOL group (Fig. 3, *A*). The in vitro stimulation increased the IL-2 production of intrahepatic $CD4^+$ T cells in both groups. Interestingly, intrahepatic $CD4^+$ T cells of the TOL group isolated at different times (days 30, 60, and 100) in the late phase after transplantation also exhibited this quality (not shown).

IL-4 production was detectable in the early phase after transplantation in the REJ group as well as in the TOL group. In the REJ model, freshly isolated intrahepatic $CD4^+$ T cells produced IL-2 (see Fig. 3, *A*) as well as IL-4 (Fig. 3, *B*) in the early phase after transplantation, showing that the inflammatory situation is not influenced by Th1 cytokines alone. Levels of secreted IL-4 detectable in supernates after 3 hours' incubation without stimulation were higher in the REJ group than in the TOL group. As described for IL-2, again the in vitro restimulation increased cytokine production.

Intrahepatic $CD4^+$ T cells differ in their ability to produce IL-13 in response to stimulation in the early and late phases after transplantation. As shown in Fig. 2, the ratio of intrahepatic $CD45RC^-$ to $CD45RC^+$ $CD4^+$ T cells changed dramatically between days 3 and 5 and between days 14 and 30 in the TOL group compared to the SYN rats. After day 30, the ratio of $CD45RC^-/CD45RC^+$ $CD4^+$ T cells continuously decreased again until day 100, so that the $CD4^+$ T-cell population isolated on days 30, 60, and 100 showed a dominance of T cells with the $CD45RC^+$ phenotype. T cells isolated at these time points were able to express IL-13 mRNA in response to in vitro stimulation (Fig. 4). $CD4^+$ T cells isolated from syngeneic liver grafts did not express such levels of IL-13 mRNA after stim-

ulation as CD4+ T cells from the TOL group did. This suggests that after liver transplantation, a defined T-cell population shows differences in its ability to produce cytokines in response to stimulation at different times after transplantation. These intragraft Th2-like CD4+ T cells may have a regulatory function in the induction of liver tolerance. In contrast, the IL-10 production of these cells did not differ from that of CD4+ T cells isolated from syngeneically transplanted livers (not shown).

DISCUSSION

The incompatibility of the MHC between the foreign organ and the recipient constitutes a major barrier to allograft acceptance,[18] because the donor organ is rejected unless the recipient is given immunosuppressants. Liver allografts break this paradigm because, despite a complete MHC mismatch, they are often not rejected, even in the absence of immunosuppression.[19] There are published observations that appear to indicate that intragraft passenger leukocytes are required for this spontaneous form of liver acceptance.[20,21] The mechanism, however, is not known in detail. In this study we differentiated between the two intrahepatic CD4+ T-cell subpopulations, CD45RC+ and CD45RC−, by analyzing the changes in their ratios after transplantation. Several studies indicate that the switch from CD45RC+ to CD45RC− (activated or memory) cells is induced by antigen encounter.[22] These studies indicate that the level of expression of CD45RC correlates with resting and/or naive (CD45RC+) T cells and activated or memory (CD45RC−) T cells.[8] CD45RC−/CD45RC+ CD4+ T cells increased in the early phase after transplantation, and this increase correlates with elevated levels of activated (IL-2 receptor positive) T cells, an indication that this phase is characterized by an inflammatory process based mainly on the activation of CD4+ T cells. This indicates that liver allograft tolerance in the TOL group was associated with an intrahepatic activation process in the early phase between days 3 and 7 and between days 14 and 30. After day 30, the levels of activated (CD45RC−) CD4+ T cells decreased continuously. In response to the 3-hour in vitro stimulation with monoclonal antibodies R7.3 and JJ319, these isolated CD4+ T cells exhibited increased mRNA levels of the Th2 cytokine IL-13. Intrahepatic CD4+ T cells of the SYN control rats showed a constant CD45RC−/CD45RC+ ratio over the same time, and these cells were not able to produce increased amounts of IL-13 mRNA after restimulation.

Although different immune mechanisms are involved in the rejection reaction of vascularized allografts, CD4+ T cells play a predominant role in triggering and controlling this process. However, distinct regulatory subpopulations of CD4+ T cells are able to control the allogeneic immune response and are therefore able to induce nonresponsiveness in alloreactive T cells. Since Bretscher and Cohn[23] first defined T-cell tolerance mechanisms in terms of deletion and anergy in 1970,[23] it has been clear that distinct regulatory T cells are relevant to the control of alloimmune responses. CD4+ T cells affect the alloresponse toward rejection or tolerance through the production of different cytokine profiles. Studies using animal transplant models have clearly demonstrated their involvement in both processes.[25] Accumulated data support the role of immunosuppressive CD4+ T cells, although the precise mechanism underlying their ability to prevent allograft rejection is presently unknown.[26] It has been suggested that immune deviation toward a Th2 response may be involved[27]; however, direct evidence that Th2 cells are responsible for maintaining survival of allografts has not been reported.[28] In some situations, however, Th2 cell involvement has a detrimental effect, for example, in the development of chronic rejection.[29]

Cytokines are small soluble proteins that are important mediators of the immune system. They regulate a variety of immune and locally inflammatory responses by affecting proliferation, differentiation, and the functioning of the various immunocompetent cells involved in these processes. Quantitation of cytokines involved in tolerance induction is essential for gaining insight into the local immune process of the liver allograft. At present, it is not clear whether IL-13 is involved in tolerance induction of liver allografts, although it shares many biologic functions with IL-4. IL-4–producing regulatory Th2-like CD4+ T cells have been identified as mediators of tolerance in some cases.[30] Both cytokines inhibit the production of inflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor-alpha, and thus negatively regulate the inflammatory process.[31]

For clinical liver transplantation, identification of the precise mechanisms underlying liver allograft tolerance is essential to minimize or avoid the need for immunosuppressive treatment of patients undergoing liver transplantation. Identification of the nature of regulatory cells that mediate tolerance induction is thus a major goal in the field of transplantation immunology. Immunoregulatory T cells may also be an important diagnostic tool for monitoring the tolerance process. Further investigations are necessary to clarify the function of the IL-13–producing intrahepatic CD4+ T cells described here.

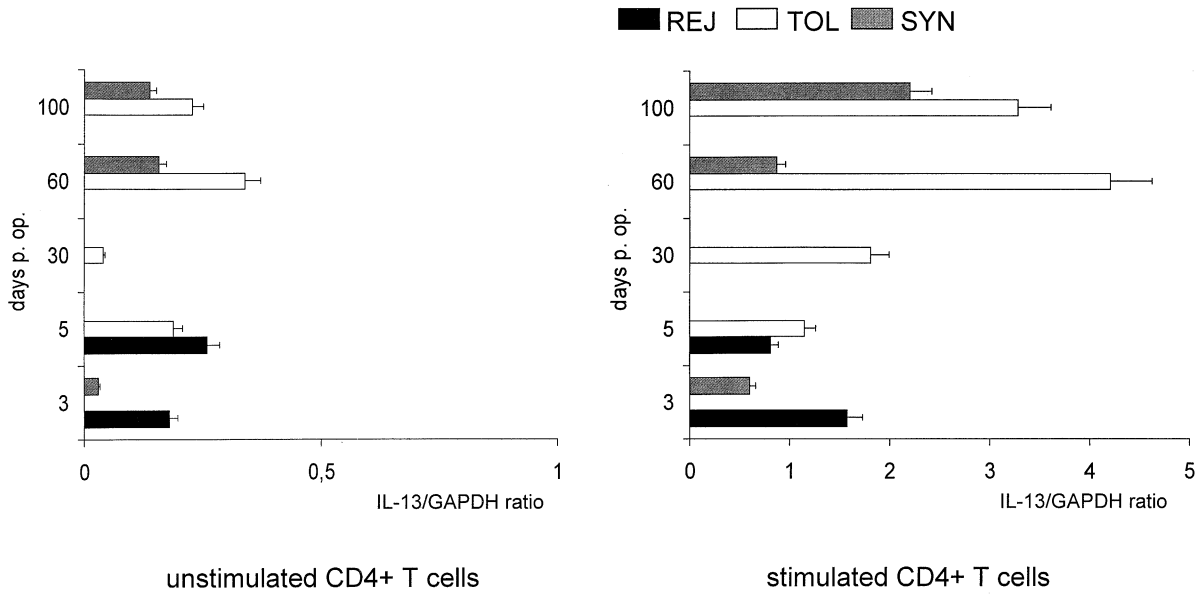


Fig. 4. Semiquantitative analysis of IL-13 mRNA expression of nonstimulated and stimulated $CD4^+$ T cells in the tolerance (*TOL*), rejection (*REJ*), and syngeneic (*SYN*) control groups at different times after transplantation. Total RNA was isolated from 6×10^5 $CD4^+$ T cells and analyzed by RT-PCR as described in Material and Methods. Results are expressed as the ratio between the optical density of IL-13 amplicon and the optical density of the housekeeping gene GAPDH. Data are means ± standard deviation of RNA samples from at least two animals per time point.

It is not clear whether these cells are present in the liver allograft only or if they also occur in the peripheral blood system. It is important that these cells and their products be characterized to enable their use for therapeutic purposes.

CONCLUSION

In the present study we examined the cytokine production of intrahepatic $CD4^+$ T cells isolated at different times after transplantation to identify possible regulatory T cells according to their cytokine production. After day 30, the intrahepatic T-cell population was able to produce increased mRNA levels of the Th2 cytokine IL-13 after stimulation with anti-TCR and anti-CD28 monoclonal antibodies in vitro. This is in contrast to the ability of $CD4^+$ T cells isolated on day 3 after transplantation; they did not produce IL-13 after in vitro stimulation. It appears that the intrahepatic $CD4^+$ T cells isolated in the early and late phases after transplantation differ in their ability to induce type 1 and type 2 immune responses. The presence of these possibly immunoregulatory cells in the late phase after transplantation seems to be characteristic of livers that are not rejected. Future studies will attempt to characterize the functional importance of these intrahepatic T cells in the late phase after transplantation, especially their ability to secrete a Th2-dominated cytokine profile.

We gratefully acknowledge the skillful technical assistance of Mrs. J. Grimmer.

REFERENCES

1. Cattral MS, Lilly LB, Levy GA. Immunosuppression in liver transplantation. Semin Liver Dis 2000;20:523–531.
2. Davies JD, Martin G, Phillips J, Marshall SE, Cobbold SP, Waldmann H. T cell regulation in adult transplantation tolerance. J Immunol 1996;157:529–533.
3. Qin S, Cobbold SP, Pope H, Elliott J, Kioussis D, Davies J, Waldmann H. Infectious transplantation tolerance. Science 1993;259:974–977.
4. Weigle WO, Romball CG. CD4+ T-cell subsets and cytokines involved in peripheral tolerance. Immunol Today 1997;18:533–538.
5. Levings MK, Sangregorio R, Galbiati F, Squadrone S, de Waal Malefyt R, Roncarolo MG. IFN-α and IL-10 induce the differentiation of human type 1 T regulatory cells. J Immunol 2000;166:5530–5539.
6. Powrie F, Mason D. Subsets of rat CD4+ T cells defined by their differential expression of variants of the CD45 antigen: Developmental relationships and in vitro and in vivo functions. Curr Top Microbiol Immunol 1990;159:79–96.
7. McKnight AJ, Barclay AN, Mason DW. Molecular cloning of rat interleukin 4 cDNA and analysis of the cytokine repertoire of subsets of CD4+ T cells. Eur J Immunol 1991;21:1187–1194.

8. Yang CP, Bell EB. Functional maturation of recent thymic emigrants in the periphery: Development of alloreactivity correlates with the cyclic expression of CD45RC isoforms. Eur J Immunol 1992;22:2261–2269.
9. Sun J, Sheil AG, Wang C, Wang L, Rokahr K, Sharland A, Jung SE, Li L, McCaughan GW, Bishop GA. Tolerance to rat liver allografts: IV. Acceptance depends on the quantity of donor tissue and on donor leukocytes. Transplantation 1996;62:1725–1730.
10. Gassel HJ, Steger U. Liver transplantation. In Timmermann W, Gassel H-J, Ulrichs K, Zhong R, Thiede A, eds. Organ Transplantation in Rats and Mice. Berlin: Springer Verlag, 1998, p 123.
11. Ono K, Lindsey ES. Improved technique of heart transplantation in rats. J Thorac Cardiovasc Surg 1969;57:225–229.
12. Jefferies WA, Green JR, Williams AF. Authentic T helper CD4 (W3/25) antigen on rat peritoneal macrophages. J Exp Med 1985;162:117–127.
13. Spickett GP, Brandon MR, Mason DW, Williams AF, Woollett GR. MRC OX-22, a monoclonal antibody that labels a new subset of T lymphocytes and reacts with the high molecular weight form of the leukocyte-common antigen. J Exp Med 1983;158:795–810.
14. Siegling A, Lehmann M, Platzer C, Emmrich F, Volk HD. A novel multispecific competitor fragment for quantitative PCR analysis of cytokine gene expression in rats. J Immunol Methods 1994;177:23–28.
15. Kruse JJ, Bart CI, Leer JW, Wondergem J. Detection and quantitative measurement of transforming growth factor-beta1 (TGF-beta1) gene expression using a semi-nested competitive PCR assay. Cytokine 1999;11:179–185.
16. Gillespie KM, Saoudi A, Kuhn J, Whittle CJ, Druet P, Bellon B, Mathieson PW. Th1/Th2 cytokine gene expression after mercuric chloride in susceptible and resistant rat strains. Eur J Immunol 1996;26:2388–2392.
17. VanderBorght A, van der Aa A, Geusens P, Vandevyver C, Raus J, Stinissen P. Identification of overrepresented T cell receptor genes in blood and tissue biopsies by PCR-ELISA. J Immunol Methods 1999;223:47–61.
18. Klein J, Sato A. The HLA system. Second of two parts. N Engl J Med 2000;343:782–786.
19. Bishop GA, Sun J, Sheil AG, McCaughan GW. High-dose/activation-associated tolerance: A mechanism for allograft tolerance. Transplantation 1997;64:1377–1382.
20. Sun J, McCaughan GW, Gallagher ND, Sheil AG, Bishop GA. Deletion of spontaneous rat liver allograft acceptance by donor irradiation. Transplantation 1995;60:233–236.
21. Shimizu Y, Goto S, Lord R, Vari F, Edwards-Smith C, Chiba S, Schlect D, Buckley M, Kusano M, Kamada N. Restoration of tolerance to rat hepatic allografts by spleen-derived passenger leukocytes. Transpl Int 1996;9:593–595.
22. Sarawar SR, Sparshott SM, Sutton P, Yang CP, Hutchinson IV, Bell EB. Rapid re-expression of CD45RC on rat CD4 T cells in vitro correlates with a change in function. Eur J Immunol 1993;23:103–109.
23. Bretscher P, Cohn M. A theory of self-nonself discrimination. Science 1970;169:1042–1049.
24. Weigle WO, Romball CG. CD4+ T-cell subsets and cytokines involved in peripheral tolerance. Immunol Today 1997;11:533–538.
25. Baan CC, Weimar W. Intragraft cytokine gene expression: Implications for clinical transplantation. Transpl Int 1998;11:169–80.
26. Cobbold S, Waldmann H. Infectious tolerance. Curr Opin Immunol 1998;10:518–524.
27. Scully R, Cobbold SP, Mellor AL, Wissing M, Arnold B, Waldmann H. A role for Th2 cytokines in the suppression of CD8+ T cell-mediated graft rejection. Eur J Immunol 1997;27:1663–1670.
28. Piccotti JR, Chan SY, VanBuskirk AM, Eichwald EJ, Bishop DK. Are Th2 helper T lymphocytes beneficial, deleterious, or irrelevant in promoting allograft survival? Transplantation 1997;63:619–624.
29. Shirwan H. Chronic allograft rejection. Do the Th2 cells preferentially induced by indirect alloantigen recognition play a dominant role? Transplantation 1999;68:715–726.
30. Field EH, Gao Q, Chen NX, Rouse TM. Balancing the immune system for tolerance: a case for regulatory CD4 cells. Transplantation 1997;64:1–7.
31. Minty A, Chalon P, Derocq JM, Dumont X, Guillemot JC, Kaghad M, Labit C, Leplatois P, Liauzun P, Miloux B, et al. Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. Nature 1993;362:248–250.

Geranylgeranylacetone Suppresses Inflammatory Responses and Improves Survival After Massive Hepatectomy in Rats

Hironobu Oda, M.D., Hidenori Miyake, M.D., Takashi Iwata, M.D., Kenji Kusumoto, M.S., Kazuhito Rokutan, M.D., Seiki Tashiro, M.D.

Overproduction of heat shock protein 70 (HSP70) in the liver protects hepatocytes under various pathologic conditions. In this study we examined the effects of a nontoxic HSP70 inducer, geranylgeranylacetone (GGA), on acute hepatic failure after 95% hepatectomy in rats. When GGA (100 mg/kg) or vehicle was intragastrically administered to rats 4 hours before 95% hepatectomy, all 25 rats pretreated with vehicle died within 60 hours after the operation, whereas 10 of 25 rats pretreated with GGA survived. During the 24-hour postoperative period, GGA significantly suppressed the release of aspartate or alanine aminotransferase and elevation of the serum interleukin-6 level, and completely inhibited an increase in the serum level of tumor necrosis factor-alpha. Histologic examinations showed that GGA prevented hemorrhagic necrosis, which was observed in vehicle-treated livers more than 12 hours after the operation. During the 24-hour postoperative period, HSP70 induction was absent in remnant livers of vehicle-treated rats. In contrast, GGA stimulated the HSP70 mRNA expression and HSP70 accumulation within 4 hours, and viable hepatocytes contained abundant HSP70 in their nuclei. Our results suggest that GGA may prevent acute liver failure after massive hepatectomy, at least in part, by enhancing HSP70 induction in the remnant liver. (J GASTROINTEST SURG 2002;6:464–473.)

KEY WORDS: Massive hepatectomy, liver failure, geranylgeranylacetone, heat shock protein 70

Recent advances in surgical procedures and perioperative management have made it possible to facilitate aggressive, extended hepatectomy for radical treatment of malignant liver tumors. However, management of postoperative liver failure is still one of the most challenging problems. The liver has a high regeneration potential. Surgical removal of more than one third of the liver results in a highly synchronized cell reentry pattern, reaching peak S-phase activity at 24 to 48 hours, maximal mitosis at 72 hours, and cessation of cell proliferation at 96 hours.[1] Rats undergoing greater than 90% hepatectomy have been known as a model of acute, fatal liver failure,[2,3] and most of these rats die within 24 hours and cannot survive until the onset of regeneration. However, once they have passed through this acute phase, their livers may be able to reestablish normal organ histology.

Acute hepatic failure after massive hepatectomy may be simply explained by hemodynamic and metabolic overloading on the extremely small mass of residual, functional liver. In fact, portal-systemic shunts have been shown to significantly improve the early survival rate.[4] The overload-induced damage was considered to be aggravated additionally by many factors such as disturbances of hepatic microcirculation,[5] endotoxemia,[5–7] and overproduction of inflammatory cytokines[8–10] and reactive oxygen intermediates,[11] leading us to believe that distinct agents that protect hepatocytes against the insult-induced damage during the critical period may improve the survival rate after massive hepatectomy.

A stress-inducible heat shock protein 70 (HSP70) is one of the best-known endogenous factors protecting cell and tissue injuries under various pathologic conditions.[12–14] Overproduction of HSP70 in

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (oral presentation).

From the First Department of Surgery (H.O., H.M., T.I., S.T.) and Department of Nutrition (K.K., K.R.), School of Medicine, The University of Tokushima, Tokushima, Japan.

Supported by a Grant-in-Aid for Scientific Research (No. 12557105) from the Japanese Ministry of Education, Science and Culture (K.R.).

Reprint requests: Kazuhito Rokutan, M.D., Department of Nutrition, School of Medicine, University of Tokushima, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan. e-mail: rokutan@nutr.med.tokushima-u.ac.jp

Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(01)00043-9

the liver induced by whole-body hyperthermia[15–18] or ischemic preconditioning[19] was shown to increase resistance to ischemia/reperfusion injury[15,16,19] and damage caused by hepatotoxic compounds.[17,18] We were particularly interested in the action of a nontoxic HSP70 inducer, geranylgeranylacetone (GGA).[20] GGA was originally introduced as a nontoxic HSP70 inducer that effectively exhibited protective actions on gastric mucosa.[20] A subsequent study showed that this compound could enhance the heat shock response and suppress ischemia/reperfusion injury in rat livers.[21] In this study we examined whether GGA could improve the survival rate of rats undergoing 95% hepatectomy.

MATERIAL AND METHODS

Animals and Reagents

The animals used in these experiments were male Wistar rats weighing 250 g. They were purchased from Charles River Japan, Inc. (Kanagawa, Japan). These rats were individually housed in wire-mesh cages in a room maintained at 23° C on a 12-hour light-dark cycle. They were allowed free access to a standard laboratory chow (Oriental Yeast Co., Tokyo, Japan) and tap water. α-Tocopherol and GGA supplemented with 2 μg/ml α-tocopherol as an antioxidant were provided by Eisai Co. (Tokyo, Japan). These agents were stored at 4° C in brown glass vials filled with nitrogen gas until they were used. The animals were handled and cared for in accordance with guidelines set forth by the National Institutes of Health. The experiments and procedures were approved by the Animal Care and Use Committee of the University of Tokushima.

Administration of GGA and Hepatectomy

After an overnight fast, animals were given GGA (100 mg/kg body weight; as emulsion with 5% gum arabic and 0.004% α-tocopherol) or vehicle (5% gum arabic emulsion containing 0.004% α-tocopherol). GGA or vehicle was administered intragastrically in a volume of 5 ml/kg by means of a metal tube attached to a 5 ml syringe at 10 A.M.

Surgical procedures were performed at 2 P.M. under general anesthesia with diethyl ether. Removal of 95% of the hepatic tissue was performed by the method of Higgins and Anderson,[22] with minor modifications as described by Kubota et al.[23] Hepatic arteries and portal vein branches entering lobes to be resected were ligated and divided. To avoid bleeding from the cut wedge of the liver parenchyma, the pedicles of the lobes were transfixed with 3–0 nylon sutures before the hepatic lobes were resected. With this method, the left lobe, median lobe, right lobe, and anterior portion of the caudate lobe were sequentially removed without constricting the inferior vena cava or causing any bleeding. The posterior portion of the caudate lobe was left unresected. We also confirmed that this operation removed liver tissue corresponding to 95.87 ± 0.09% (n = 15) (minimum 93.79%; maximum 96.99%) of the total wet weight. All rats were given 10 ml/kg of 5% glucose solution No. 12557105 subcutaneously immediately after completion of the surgery, and all rats were allowed free access to 10% glucose solution after they recovered from the anesthesia. All surgical procedures were completed within 25 minutes. Signs of distress and survival were monitored for up to 1 week.

Sampling

Blood was collected from the inferior vena cava immediately or at 4, 8, 12, 16, and 24 hours after hepatectomy under general anesthesia with diethyl ether. The residual liver was also removed at these time points. After the wet weight of the livers was measured, they were subjected to histologic examination or Northern and Western blot analysis. Serum was immediately separated, and the activities of aspartate (AST) and alanine (ALT) aminotransferases were measured using AST and ALT assay kits (Roche, Basel, Switzerland), respectively. Serum levels of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) were measured with enzyme-linked immunosorbent assay (ELISA) kits (Bio Source International, Camarillo, California).

Measurment of HSP70 mRNA and Protein Levels

Total RNA was prepared by homogenizing liver tissues with an acid guanidinium thiocyanate-phenol-chloroform mixture (Isogen; Nippon Gene, Toyama, Japan).[20] An equal amount of RNA (20 μg per lane) was separated in a 1% agarose-formaldehyde gel and transferred to a nylon membrane filter (Hybond-N-plus; Amersham Pharmacia, Piscataway, New Jersey). Blots were prehybridized and then hybridized with a ^{32}P-labeled cDNA probe for human HSP70 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as previously described.[20] These membranes were exposed to Kodak X-Omat x-rays for an appropriate period of time at −80° C.

For immunoblot analysis, the resected tissues were immediately snap-frozen with stainless steel tongs precooled in liquid nitrogen and stored at −80° C. The frozen specimens (approximately 40 mg) were homogenized in three volumes of lysis

buffer consisting of 10 mmol/L Tris-HCl (pH 7.8), 0.5% polyethylene glycol para-isooctylphenylether, 150 mmol/L NaCl, 1 mol/L phenylmethylsulfonyl fluoride, and 0.1 U/ml aprotinin. The homogenates were centrifuged at 15,000 *g* for 15 minutes at 4° C. An equal amount of protein in the supernates (40 μg protein per lane) was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After nonspecific binding sites were blocked with 4% purified milk casein, the blots were incubated with a monoclonal antibody against HSP70 (Santa Cruz Biotech, Santa Cruz, California). Bound antibodies were detected with an enhanced chemiluminescence Western blot detection kit (Amersham Pharmacia).

Histologic Examination

Liver tissues were fixed in 10% buffered formalin and stained with hematoxylin and eosin. For immunohistochemical detection of HSP70-expressing cells, samples were fixed overnight in Bouin's solution and immersed in 50% ethanol for 24 hours and then in 70% ethanol for 24 hours. Specimens were embedded in paraffin. These specimens were cut into sections 4 μm thick. They were deparaffinized and hydrated through xylene and graded ethanol. Endogenous peroxidase activities were quenched by incubating the sections with 0.3% hydrogen peroxide in methanol for 30 minutes. After blocking nonspecific binding sites with 1% mouse serum in phosphate-buffered saline solution for 20 minutes, the sections were incubated for 1 hour with a mouse monoclonal antibody against HSP70 (StressGen Biotech, Victoria, Alberta, Canada) at room temperature. After being washed with phosphate-buffered saline, bound antibodies were visualized with an avitin-biotin-peroxidase system (Nichirei, Tokyo, Japan). A normal mouse IgG was used to assess nonspecific reactions.

Statistical Analysis

Analysis of variance and Scheffé's test were used to determine statistically significant differences. Differences were considered significant at $P < 0.05$.

RESULTS

Effects of GGA on Survival After 95% Hepatectomy

Initially we determined an optimal dose of GGA by monitoring 7-day survival rates after 95% hepatectomy. Vehicle or GGA at different doses (1, 10, 25, 50, 100, and 200 mg/kg) were intragastrically administered to five rats in each group 4 hours before the operation. As shown in Fig. 1, *A*, all of the rats pretreated with vehicle or GGA at dosages of 1 and 25 mg/kg died within 60 hours, whereas 60% of the rats pretreated with GGA at dosages of 50, 100, or 200 mg/kg survived. After this time point, rats treated with 100 mg/kg GGA appeared to show a better clinical course; therefore we chose 100 mg/kg GGA for this study.

Half of the rats (n = 25) were given GGA (100 mg/kg; as emulsion with 5% gum arabic and 0.004% α-tocopherol), and the other half (n = 25) were given vehicle (5% gum arabic emulsion containing 0.004% α-tocopherol). Four hours later, these rats were subjected to 95% hepatectomy by a blind oper-

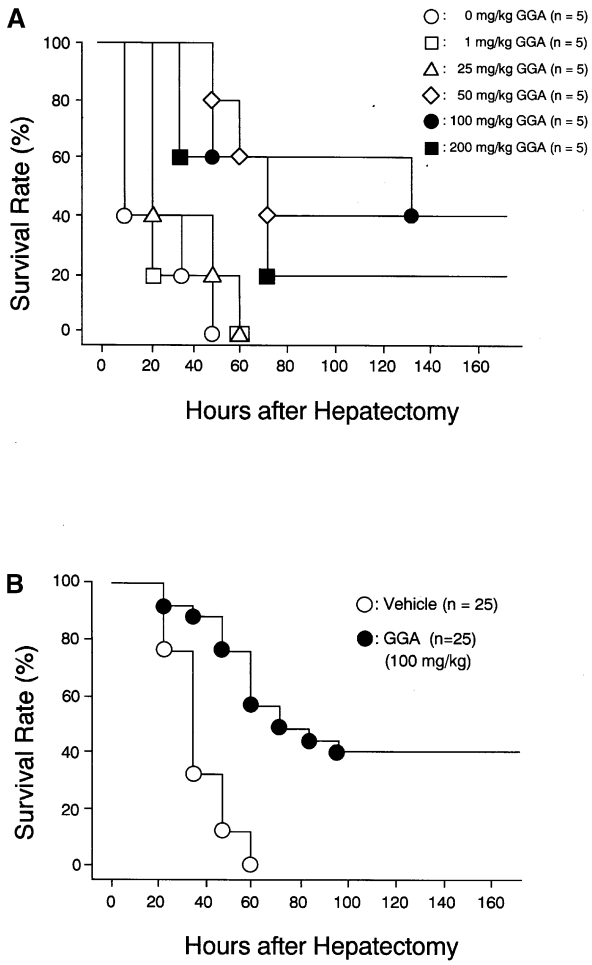


Fig. 1. Effects of GGA on survival after 95% hepatectomy. **A,** Each group of rats (n = 5 per group) was given a different dose of GGA (1, 25, 50, 100, and 200 mg/kg) or vehicle. Four hours later, 95% hepatectomy was performed, and survival was monitored for up to 160 hours. **B,** Rats pretreated with 100 mg/kg GGA (n = 25) or vehicle (n = 25) were subjected to 95% hepatectomy, and their survival rates were pursued.

ator. As shown in Fig. 1, *B*, all of the vehicle-treated rats died within 60 hours, whereas approximately 40% of the GGA-treated rats were able to survive.

Fig. 2, *A* shows the posterior portion of the caudate lobe left unresected immediately after the operation. During the 24-hour postoperative period, the wet weight of the residual liver was unchanged both in vehicle-treated and GGA-treated rats. Although approximately one third of the rats treated with GGA died within 48 hours, the liver weights began to increase at 48 hours and gradually recovered to 4.81 ± 0.11 g (n = 8) on day 7 in the GGA-treated rats. As shown in Fig. 2, *B*, the reconstituted liver showed normal histogenesis on day 7. In contrast, in vehicle-treated rats the liver weight failed to increase (Fig. 2, *C*), and all of the rats died within 60 hours.

Effects of GGA on Serum Levels of Transaminase, TNF-α, and IL-6

After 95% hepatectomy, serum AST and ALT levels in vehicle-treated rats continued to be elevated until 24 hours (Fig. 3), at which time, one-fourth of the rats died (see Fig. 1, *B*). In GGA-treated animals, serum AST levels showed a similar increase until 16 hours and then decreased at 24 hours (Fig. 3, *A*). GGA treatment significantly suppressed the elevation of the serum ALT level at 8 hours and later (Fig. 3, *B*). The magnitude of the surgical invasion was also assessed by measuring serum levels of TNF-α and IL-6. In vehicle-treated rats, 95% hepatectomy significantly increased serum levels of IL-6 (Fig. 4, *A*) and TNF-α (Fig. 4, *B*) with peaks at 8 and 16 hours, respectively. In contrast, pretreatment with GGA significantly suppressed the elevation of IL-6 at 8 and 16 hours (see Fig. 4, *A*) and almost completely inhibited TNF-α production after the surgery (see Fig. 4, *B*).

Effects of GGA on Histologic Changes After 95% Hepatectomy

After removal of 95% of the liver, congestion of the remnant liver developed rapidly and progressively. During the initial 6 to 8 hours after the surgery, vacuolation and chromatin condensation in hepatocytes were similarly observed in both GGA-treated and vehicle-treated livers. After this time, the lobular structure was later disturbed and massive hemorrhagic necrosis was observed to various extents after more than 12 hours in vehicle-treated livers (Fig. 5, *A*). Representative light micrographs with a high magnification showed that neutrophils infiltrated areas of hemorrhagic necrosis. Most of the hepatocytes were extensively vacuolated, and various sizes of eosinophilc, hyaloid bodies were observed both inside and outside of the cells (Fig. 5, *B*). In contrast, the structure of hepatic lobule remained relatively stable, and hemorrhagic necrosis was scarcely visible in GGA-treated livers (Fig. 5, *C*). Extensive vacuolation and hyaloid

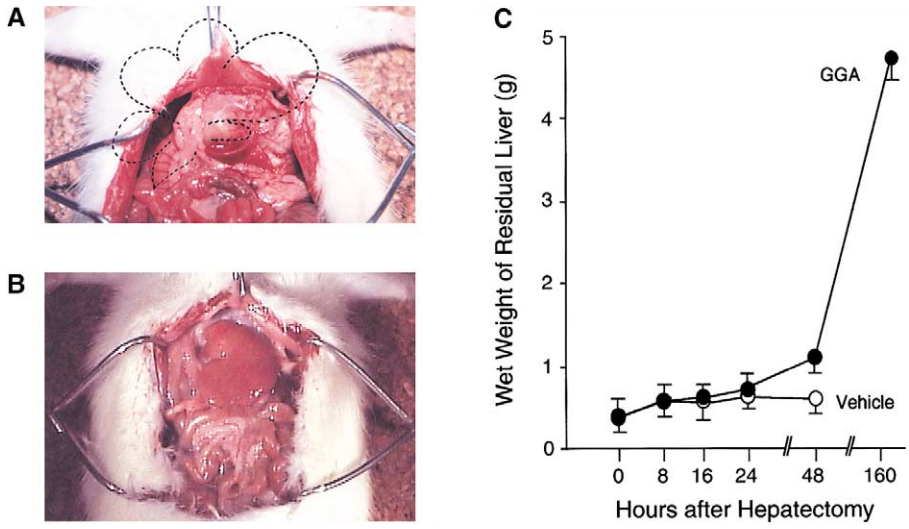
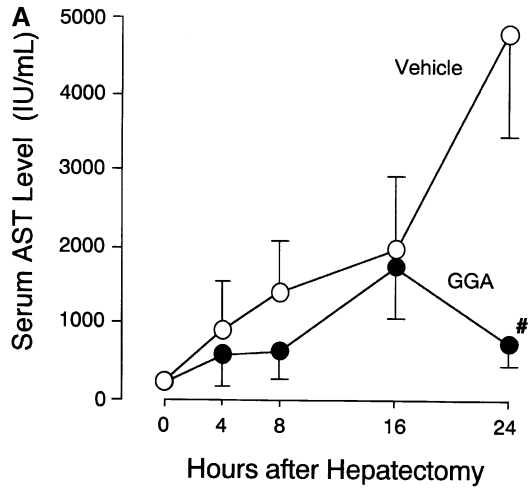


Fig. 2. Reconstitution of the liver after 95% hepatectomy. **A,** The posterior portion of the caudate lobes left unresected is shown. **B,** After 95% of the liver was removed in rats pretreated with 100 mg/kg GGA, a typical regenerated liver 7 days after the operation is shown. **C,** Changes in total wet weight of liver tissue after 95% hepatectomy are summarized. Each value is mean ± SD in the eight surviving rats, except in four vehicle-treated rats at 48 hours.



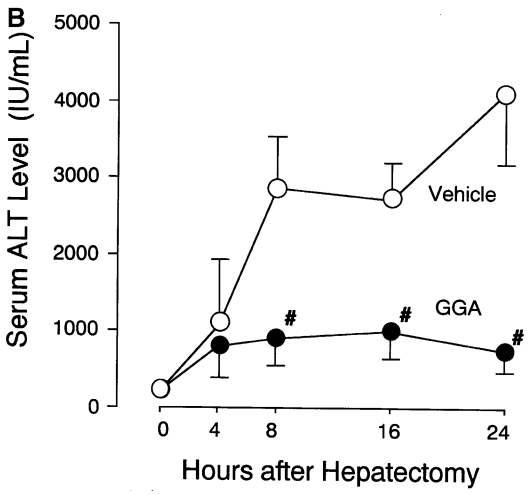
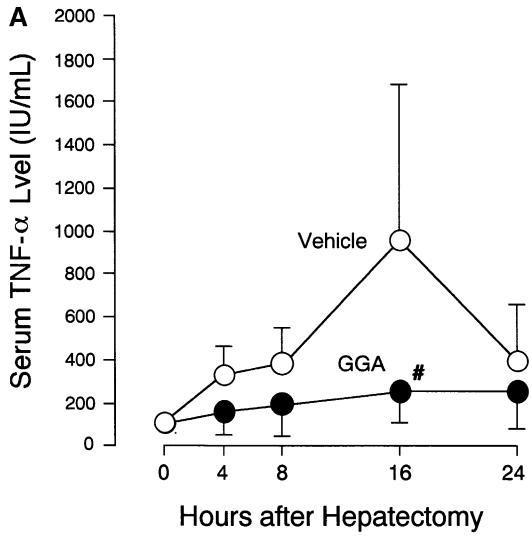


Fig. 3. Serum AST and ALT levels after 95% hepatectomy. Rats were pretreated with 100 mg/kg GGA or vehicle and subjected to 95% hepatectomy. Serum AST (**A**) and ALT (**B**) levels were measured immediately (0) or at 4, 8, 16, and 24 hours after hepatectomy. Values are mean ± SD, n = 8. #Significantly different vs. vehicle-treated cells at the respective time points ($P < 0.05$ by analysis of variance and Scheffé's test).



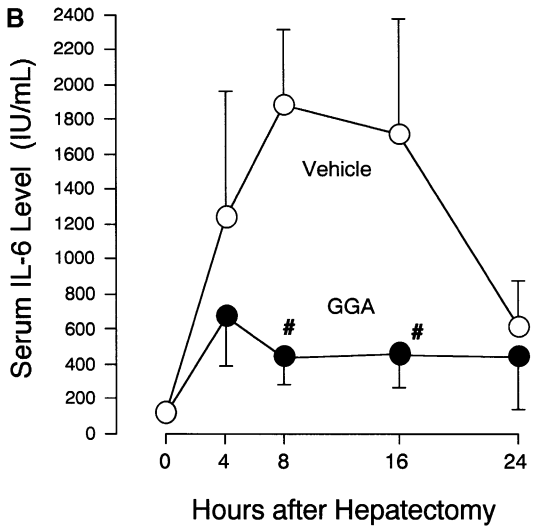


Fig. 4. Serum IL-6 and TNF-α levels after 95% hepatectomy. Serum IL-6 and TNF-α levels were measured immediately (0) or at 4, 8, 16, and 24 hours after the operation by ELISA methods as described in Material and Methods. Values are mean ± SD, n = 8. #Significantly different vs. vehicle-treated cells at the respective time points ($P < 0.05$ by analysis of variance and Scheffé's test).

body formation in hepatocytes and neutrophils was not observed in the GGA-treated tissues (Fig. 5, *D*).

Effects of GGA on HSP70 Induction After 95% Hepatectomy

In vehicle-treated livers, HSP70 mRNA expression (Fig. 6, *A*) and HSP70 induction (Fig. 6, *B*) were not observed during the 24-hour postoperative period. In contrast, significantly higher levels of HSP70 were already present in the tissue of GGA-treated rats at time 0, and pretreatment with GGA stimulated HSP70 mRNA expression (see Fig. 6, *A*) and HSP70 accumulation (Fig. 6, *B* and *C*) within 4 hours after the operation.

Immunohistochemical analysis showed that 12 hours after the surgery accumulation of HSP70 was less in the vehicle-treated tissue (Fig. 7, *A* and *B*),

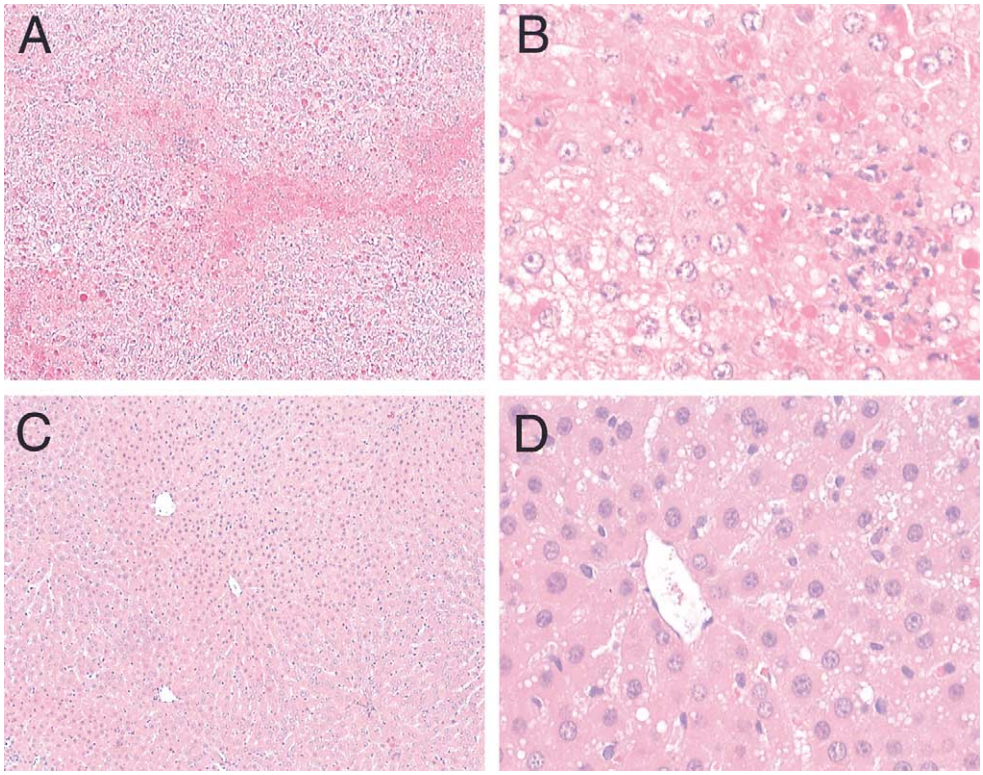


Fig. 5. Light micrographs of residual liver tissues. Residual liver tissues were removed from rats treated with vehicle (**A** and **B**) or 100 mg/kg GGA (**C** and **D**) 16 hours after 95% hepatectomy. These tissues were subjected to staining with hematoxylin and eosin. (Original magnifications: *A* and *C*, ×20; *B* and *D*, ×100.)

whereas the parenchymal hepatocytes in GGA-treated rats contained HSP70 preferentially in their nuclei as well as in cytoplasm (Fig. 7, *C* and *D*). These results were consistent with those of Western blot analysis.

DISCUSSION

Massive ablation of the rat liver (>90%) was a model for acute, lethal hepatic failure, invariably resulting in death in 100% of the animals within 40 hours without regeneration in the liver remnant.[2,3] Subsequent studies showed that a high rate of survival could be achieved by careful attention to surgical technique and blood sugar levels, and as a consequence, the minimum residual mass critical for regeneration and survival could be lowered to less than 10% of the total liver weight.[23,24] According to the method of Kubota et al.,[23] we challenged 95% hepatectomy, but all of the operated rats died within 60 hours even under the most careful management. Using this model, we examined the effects of GGA that was introduced as a nontoxic HSP70 inducer.[20]

We have already shown that GGA could directly activate heat shock factor 1 (HSF1) and induce the HSP70 mRNA expression and accumulation of HSP70 in gastric mucosal cells, both in vitro and in vivo.[20] When 125 mg/kg of [^{14}C]GGA was intragastrically administered to rats, plasma concentrations of GGA reached 10^{-6} to 10^{-5} mol/L with a peak at 3 hours, and approximately 3 μmoles/g tissue of GGA was recovered in the liver with a peak at 4 hours.[25] However, uptake by the rat liver was only 5% of the uptake by the gastric mucosa.[25] We administered GGA (200 mg/kg) intragastrically twice a day for up to 3 days, but failed to detect HSP70 induction in rat livers (date not shown). Thus GGA is not likely to be a potent HSP70 inducer for the liver, possibly because of the higher GGA-metabolizing activities (i.e., ω- and β-oxidation activities) in hepatocytes. On the other hand, Yamagami et al.[21] reported that GGA itself did not directly induce HSP70, but it markedly augmented the heat shock response in the rat liver, leading to the suppression of ischemia/reperfusion injury. More recently it has been shown that when oral administration of GGA was continued for 3 weeks (200 mg/kg twice a day), GGA enhanced HSP70 induction in transplanted

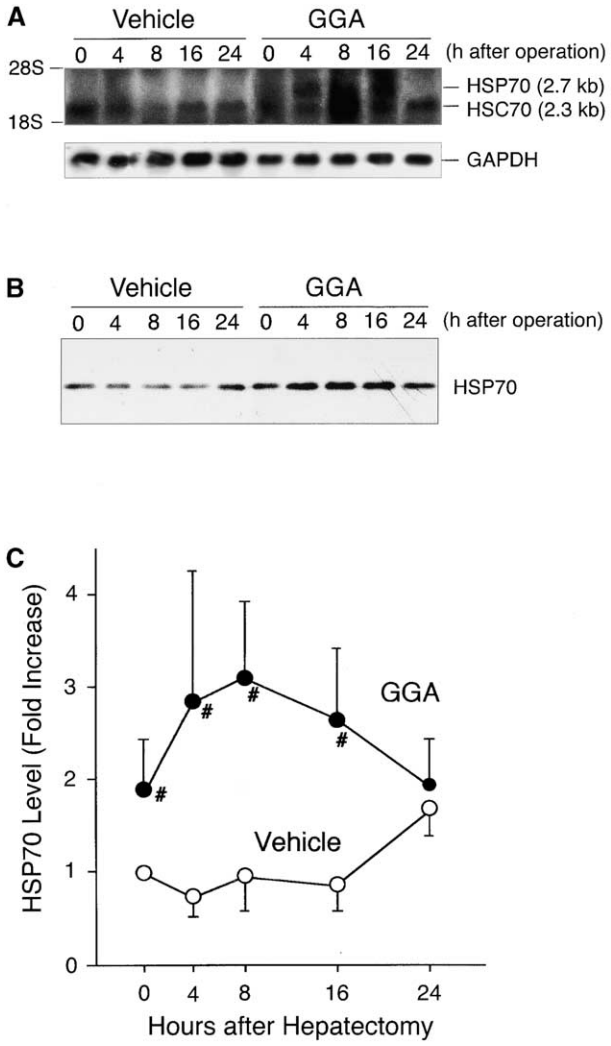


Fig. 6. Expression of HSP70 mRNA and protein after 95% hepatectomy. Total RNA and tissue protein were extracted at the indicated hours after 95% hepatectomy, as described in Material and Methods. Total RNA was subjected to Northern blot analysis with cDNA probes for human HSP70 and GAPDH. **A,** Representative changes in the HSP70 mRNA level. **B,** An equal amount of tissue protein (20 μg protein per lane) was subjected to Western blot analysis with an anti-HSP70 antibody. **C,** Levels were quantified by densitometric analysis, and each HSP70 level relative to that at time 0 in vehicle-treated tissue was calculated. Values expressed as fold increases are mean ± SD, n = 8. #Significantly different vs. vehicle-treated cells at the respective time points ($P < 0.05$ by analysis of variance and Scheffé's test).

livers and prevented warm ischemic injury in recipient rats.[26]

Based on this information, we were able to precisely evaluate the HSP70-inducing action of GGA on hepatocytes.[27] GGA was not a potent transcriptional activator of the HSP70 gene in hepatocytes. However, once hepatocytes were primed with GGA, they drastically increased their capacity for induction of HSPs, in particular HSP70, when exposed to hydrogen peroxide or ethanol. Consequently GGA-treated hepatocytes exhibited high resistance to these insults.[27] The priming effect of GGA was linked to enhanced activation of HSF1, including accelerated nuclear translocation, phosphorylation, and DNA-binding activity of HSF1.[27] These results suggest that GGA may exert a protective action on hepatocytes, as was observed in gastric mucosal cells, if GGA could be effectively delivered to the liver and concentrated therein.

GGA was slowly absorbed from the small intestine and could be concentrated in the remnant liver in our experimental model. After 95% hepatectomy, the residual tissue in vehicle-treated rats was so severely damaged that it could not induce HSP70. Even under these stressful conditions, the GGA-pretreated tissue was able to induce HSP70 during the initial 24 hours of the postoperative period. Other factors besides HSP70 may make GGA-treated hepatocytes sufficiently viable to synthesize HSP70. However, the GGA-treated tissue already contained significantly higher levels of HSP70 immediately after the operation (time 0). Once hepatocytes were primed with GGA, they could rapidly and markedly upregulate HSP70 induction.[27] Overproduction of HSP70 has been demonstrated to suppress tissue damage caused by reactive oxygen intermediates, [28] endotoxin,[29] and inflammatory cytokines such as TNF-α.[30] All of these aggravating factors have been considered to participate in acute hepatic failure after massive hepatectomy,[5–11] suggesting an important role for HSP70 in protection against this acute hepatic failure. At present, the pharmacologic actions of GGA on hepatocytes have not been fully studied; therefore other factors besides HSP70 may be involved in the protection by GGA. However, hepatocytes expressing abundant HSP70 may display higher resistance to the hepatocyte-damaging factors during the initial 24 hours of the postoperative period. There was no difference in histologic changes during the initial 8 hours, whereas GGA prevented the development of acute hemorrhagic necrosis, which was observed 16 hours or more after the operation. Consistent with the histologic differences between GGA and vehicle treatments, elevation of serum transaminase levels subsided within 24 hours in the GGA-treated tissue but not in the vehicle-treated tissue.

GGA pretreatment significantly suppressed elevation of the serum IL-6 level and almost completely inhibited TNF-α production. Recently GGA has been shown to suppress the elevation of serum

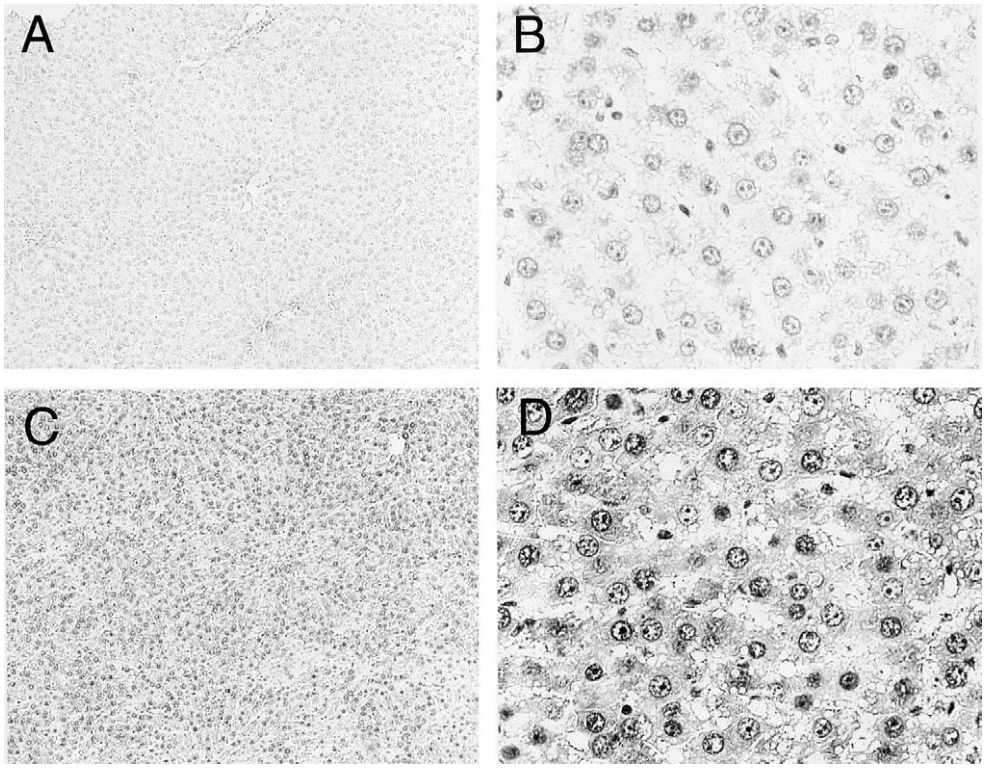


Fig. 7. HSP70-expressing cells in residual liver tissues (gastric mucosa). Rats treated with vehicle (**A** and **B**) or 100 mg/kg GGA (**C** and **D**) were subjected to 95% hepatectomy. Twelve hours after the operation, the residual tissues were subjected to immunohistochemical analysis with an anti-HSP70 antibody, as described in Material and Methods. (Original magnifications: *A* and *C*, ×20; *B* and *D*, ×100.)

TNF-α in warm ischemic injury in a rat liver transplantation model.[31] We tested whether GGA directly suppressed the activation of nuclear factor-κB in primary cultures of rat hepatocytes exposed to hydrogen peroxide or ethanol and found that GGA pretreatment did not modify the insult-induced nuclear factor-κB activation.[27] The release of IL-6 and TNF-α may simply reflect accompanying inflammation and tissue damage. It is still possible that GGA may directly suppress the activation of Kupffer cells, which are a major source of inflammatory cytokines after hepatectomy.[32] Further studies are required to address the mechanism by which GGA suppresses the cytokine release.

The present study was focused on the effects of GGA on the acute stage of the lethal hepatectomy model. Although we confirmed that GGA itself did not stimulate the growth of gastric mucosal cells and hepatocytes in vitro[33] (other data not shown), the effects of GGA on their regeneration should be examined using a nonlethal hepatectomy model. Recently several novel approaches have been applied to improve regeneration and survival rates after massive hepatectomy, such as transplantation of immortalized hepatocytes[34] and telomerase gene delivery.[35] Compared with these approaches, GGA has been widely used in Japan as an antiulcer drug for more than 15 years without any adverse effects. The present study suggests that this nontoxic HSP70 inducer may provide a therapeutic benefit for aggressive, extended hepatectomy for radical treatment of malignant liver tumors.

REFERENCES

1. Diehl AM, Rai RM. Liver regeneration 3: Regulation of signal transduction during liver regeneration. FASEB J 1996;10:215–227.
2. Tuczek HV, Rabes H. Verlust der Proliferationsfähigkeit der Hepatozyten nach subtotalen Hepatektomie. Experientia 1971;27:526.
3. Emond J, Capron-Laudereay M, Meriggi F, Bernuau J, Reynes M, Houssin D. Extent of hepatectomy in the rat: Evaluation of basal condition and effect of therapy. Eur Surg Res 1989;21:251–259.
4. Fukuchi T, Hirose H, Onitsuka A, Hayashi M, Senga S, Imai N, Shibata M, Yamauchi K, Futamura N, Sumi Y. Effects of portal-systemic shunt following 90% partial hepatectomy in rats. J Surg Res 2000;89:126–131.
5. Mochida S, Ogata I, Hirata K, Hirata K, Ohta Y, Yamada S, Fujiwara K. Provocation of massive hepatic necrosis by endotoxin after partial hepatectomy in rats. Gastroenterology 1990;99:771–777.

6. Gallery MP, Mangino MJ, Flye MW. A biological basis for limited Kupffer cell reactivity to portal-derived endotoxin. Surgery 1991;110:221–230.
7. Wang XD, Soltesz V, Andersson R, Bengmark S. Bacterial translocation in acute liver failure induced by 90% hepatectomy in the rat. Br J Surg 1993;80:66–71.
8. Calley MP, Kamai T, Flye MW. Kupffer cell tumor necrosis factor–α production is suppressed during liver regeneration. J Surg Res 1991;50:515–519.
9. Leist M, Gantner F, Bohlinger I, Tiegs G, Germann PG, Wendel A. Tumor necrosis factor-induced hepatocyte apoptosis precedes liver failure in experimental murine shock models. Am J Pathol 1995;146:1220–1234.
10. Panis Y, McMullan DM, Emond JC. Progresive necrosis after hepatectomy and the pathophysiology of liver failure after massive resection. Surgery 1997;121:142–149.
11. Andiran F, Ayhan A, Tanyel FC, Abbasoglu O, Sayek I. Regenerative capacities of normal and cirrhotic livers following 70% hepatectomy in rats and the effect of α-tocopherol on cirrhotic regeneration. J Surg Res 2000;89:184–188.
12. Welch WJ. Mammalian stress response: Cell physiology, structure/function of stress protein, and implications for medicine and disease. Physiol Rev 1992;72:1063–1081.
13. Minowada G, Welch WJ. Clinical implications of the stress response. J Clin Invest 1995;95:3–12.
14. Jaattela M. Heat shock proteins as cellular lifeguards. Ann Med 1999;31:261–271.
15. Saad S, Kanai M, Awane M, Yamamoto Y, Morimoto T, Isselhard W, Minor T, Troidl H, Ozawa K, Yamaoka Y. Protective effect of heat shock pretreatment with heat shock protein induction before hepatic warm ischemic injury caused by Pringle's maneuver. Surgery 1995;118:510–516.
16. Yamagami K, Yamamoto Y, Kume M, Kimoto S, Yamamoto H, Ozaki N, Yamamoto M, Shimahara Y, Yamaoka Y. Heat shock preconditioning ameliorates liver injury following normothermic ischemia-reperfusion in steatotic rat livers. J Surg Res 1998;79:47–53.
17. Salminen WF Jr, Voellmy R, Roberts SM. Protection against hepatotoxicity by a single dose of amphetamine: The potential role of heat shock protein induction. Toxicol Appl Pharmacol 1997;147:247–258.
18. Fujimori S, Otaka M, Otani S, Jin M, Okuyama A, Itoh S, Iwabuchi A, Sasahara H, Itoh H, Tashima Y, Masamune O. Induction of a 72-kDa heat shock protein and cytoprotection against thioacetamide-induced liver injury in rats. Dig Dis Sci 1997;42:1987–1994.
19. Kume M, Yamamoto Y, Saad S, Gomi T, Kimoto S, Shimabukuro T, Yagi T, Nakagami M, Takeda Y, Morimoto T, Yamaoka Y. Ischemic preconditioning of the rat liver in rats: Implication of heat shock protein induction to increase tolerance of ischemia-reperfusion injury. J Lab Clin Med 1996;128:251–258.
20. Hirakawa T, Rokutan K, Nikawa T, Kishi K. Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. Gastroenterology 1996;111:345–357.
21. Yamagami K, Yamamoto Y, Ishikawa Y, Yonezawa K, Toyokuni S, Yamaoka Y. Effects of geranyl-geranyl-acetone administration before heat shock preconditioning for conferring tolerance against ischemia-reperfusion injury in rat livers. J Lab Clin Med 2000;135:465–475.
22. Higgins GM, Anderson RM. Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. Arch Pathol 1931;12:186–202.
23. Kubota T, Takabe K, Yang M, Sekido H, Endo I, Ichikawa Y, Togo S, Shimada H. Minimum sizes for remnant and transplanted livers in rat. J Hepatobiliary Pancreat Surg 1997;4:398–404.
24. Gaub J, Iversen J. Rat liver regeneration after 90% partial hepatectomy. Hepatology 1984;4:902–904.
25. Nishizawa Y, Tanaka H, Kinoshita K. Absorption, distribution, metabolism, and secretion of tetraprenyacetone in rats and guinea pigs. Prog Med 1983;3:1029–1035.
26. Fudaba Y, Tashiro H, Ohdan H, Miyata Y, Shibata S, Shintaku S, Nishihara M, Asahara T, Ito H, Fukuda Y, Dohi K. Prevention of warm ischemic injury in rat liver transplantation by geranylgeranylacetone. Transplant Proc 2000; 32:1615–1616.
27. Ikeyama S, Kusumoto K, Miyake H, Rokutan K, Tashiro S. A non-toxic heat shock protein 70 inducer, geranylgeranylacetone, suppresses apoptosis of cultured rat hepatocytes caused by hydrogen peroxide and ethanol. J Hepatol 2001;35:53–61.
28. Polla BS, Kantengwa S, Francois D, Salvioli S, Franceschi C, Marsac C, Cossarizza A. Mitochondria are selective targets for the protective effects of heat shock against oxidative injury. Proc Natl Acad Sci USA 1996;93:6458–6463.
29. Hauser GJ, Dayao EK, Wasserloos K, Pitt BR, Wong HR. HSP induction inhibits iNOS mRNA expression and attenuates hypotension in endotoxin-challenged rats. Am J Physiol 1996;271:2529–2535.
30. Kim Y-M, de Vera ME, Watkins SC, Billiar TR. Nitric oxide protects cultured rat hepatocytes from tumor necrosis factor-alpha-induced apoptosis by inducing heat shock protein 70 expression. J Biol Chem 1997;272:1402–1411.
31. Fudaba Y, Tashiro H, Ohdan H, Miyata Y, Shibata S, Shintaku S, Nishihara M, Asahara T, Ito H, Fukuda Y, Dohi K. Efficacy of HSP72 induction in rat liver by orally administered geranylgeranylacetone. Transpl Int 2000;13:S278–S281.
32. Rai RM, Yang SQ, McClain C, Karp CL, Klein AS, Diehl AM. Kupffer cell depletion by gadolinium chloride enhances liver regeneration after partial hepatectomy in rats. Am J Physiol 1996;270:909–918.
33. Rokutan K, Teshima S, Kawai T, Kawahara T, Kusumoro K, Mizushima T, Kishi K. Geranylgeranylacetone stimulates mucin synthesis in cultured guinea pig gastric pit cells by inducing a neuronal nitric oxide synthase. J Gastroenterol 2000;35:673–681.
34. Kobayashi N, Fujiwara T, Westerman KA, Inoue Y, Sakaguchi M, Noguchi H, Miyazaki M, Cai J, Tanaka N, Fox IJ, Leboulch P. Prevention of acute liver failure in rats with reversibly immortalized human hepatocytes. Science 2000; 287:1258–1262.
35. Rudolph KL, Chang S, Millard M, Schreiber-Agus N, DePinho A. Inhibition of experimental liver cirrhosis in mice by teromerase gene delivery. Science 2000;287:1253–1258.

Discussion

Dr. K. Behrns (Chapel Hill, NC): You nicely showed yesterday that in primary hepatocytes GGA is involved with the apoptosis and the caspases, but this model has hemorrhagic necrosis. So what do you think are the operative pathways that allow GGA protection?

Dr. H. Oda: GGA can prevent both necrosis and apoptosis because necrosis and apoptosis must share common targets against insults in the very early stage. So I think GGA can protect these common targets.

Dr. Behrns: How about the JNK pathway?

Dr. Oda: In this model, damage was so severe that we could not evaluate apoptotic pathways, such as activation of JNK.

Dr. E. Klar (Heidelberg, Germany): You placed a great deal of emphasis on the induction of heat shock protein, but that is expressed only a couple of hours after drug administration. So I am wondering why you chose only 4 hours of time before the main experiment to administer GGA and why you did not go further forward 6 or 12 hours to reach a heat shock protein expression peak at the time when you induced trauma? Why was it not applied earlier?

Dr. Oda: After an oral administration, we found that GGA was slowly accumulated in the liver with a peak at 4 hours.

Dr. Klar: But after hepatic ischemia/reperfusion injury, we saw that heat shock protein is expressed much later. Only heat shock protein mRNA is expressed within 4 hours.

Dr. K. Rokutan: I am a coauthor. As to the first question, we determined what the effective period was to enhance HSP70 induction in the liver after oral administration, and we found that 4 hours of pretreatment was the best to effectively enhance the HSP70 induction after surgery. As to the second question, GGA mainly exerted protective actions by priming livers for enhanced HSP70 induction after the hepatectomy. Four-hour treatment with GGA already induced a significant amount of HSP70 before the operation and markedly enhanced HSP70 induction during the acute phase of the postoperative period. That is the best condition for treatment with GGA.

Invited Discussion—Expert Commentator

Henry A. Pitt, M.D. (Milwaukee, WI): This paper demonstrates in a rat model of post-hepatectomy liver failure that a single preoperative dose of geranylgeranylacetone, GGA, improves survival by enhancing heat shock protein response. This paper adds to the ongoing body of data suggesting that protection of ischemia-reperfusion injury improves outcome. This paper also confirms that the ischemic preconditioning strategy, which also induces heat shock protein, may be wise.

Like many good studies, this paper asks more questions than it answers. For example, would multiple preoperative doses of GGA be better than one? Would the administration of GGA for two to three weeks be better than a short preoperative dose? Would postoperative GGA be beneficial? The authors also state in their discussion that GGA has been used extensively in humans in Japan for the treatment of ulcers without toxicity. However, the question remains as to whether administration of GGA is safe in patients with obstructive jaundice and/or underlying liver disease who may require a major liver resection.

Pancreatic Elastase Induces Liver Injury by Activating Cytokine Production Within Kupffer Cells via Nuclear Factor-Kappa B

Michel M. Murr, M.D., Jun Yang, M.D., Adam Fier, B.A., Pam Kaylor, M.S., Stephen Mastorides, M.D., James G. Norman, M.D.*

Liver injury is a manifestation of the systemic inflammatory response during acute pancreatitis. We have demonstrated that elastase induces macrophage tumor necrosis factor (TNF) production in distant organs, thus mimicking pancreatitis-associated organ injury. The aim of this study was to determine the mechanism by which elastase induces hepatic cytokine production. Rat livers (n = 40) were perfused with elastase ± gadolinium (Gd) to inhibit Kupffer cells. Liver parenchymal enzymes and TNF were measured in the effluent. In vitro, rat hepatocytes or Kupffer cells were treated with elastase (1 U/ml) ± Gd (0.5 mg/ml) or pyrrolidine dithiocarbamate (PDTC; 0.5 mg/ml). TNF protein, TNF messenger RNA, and NF–κB activation were determined. In vivo, Gd blunted the elastase-induced TNF production and decreased AST, ALT, LDH, and nonviable cells (propidium iodide) ($P \leq 0.03$ vs. elastase). In vitro, elastase induced TNF production from Kupffer cells ($P < 0.001$ vs. control) but not from hepatocytes. Gd or PDTC significantly attenuated the elastase-induced TNF production ($P < 0.001$). Elastase-induced overexpression of TNF messengerRNA and activation of NF-κB was attenuated by Gd. Pancreatic elastase induces a pattern of liver injury similar to that seen during acute pancreatitis by activating cytokine production and gene expression within Kupffer cells via NF-κB. Gd exhibits a protective effect against elastase-induced liver injury by inhibiting activation of NF-κB. (J GASTROINTEST SURG 2002;6:474-480.)

KEY WORDS: Pancreatitis, pancreatic enzymes, liver injury, cytokines, Kupffer cells

Liver injury is a manifestation of the systemic inflammatory response during acute pancreatitis and an important clinical prognostic indicator in that setting. The morbidity and mortality associated with severe acute pancreatitis are largely attributable to exacerbation of the systemic inflammatory response and the subsequent distant organ dysfunction.

We have demonstrated that pancreatic elastase plays a major role in extrapancreatic, organ-specific cytokine production, thus suggesting that pancreatic elastase is the link between localized inflammation of the pancreas and the systemic manifestations of pancreatitis and distant organ injury.[1–4] In addition, work from our laboratory has demonstrated that acute pancreatitis–associated liver injury is mediated by noxious inflammatory cytokines that are produced within tissue resident macrophages.[4,5] In that regard, the liver is a unique organ because Kupffer cells are the largest population of fixed tissue macrophages and have been shown to have a distinct role in sepsis and hemorrhage.[6–8] However, the role of Kupffer cells in the pathogenesis of pancreatitis-associated liver injury is poorly understood and warrants further investigation. The current study was undertaken to determine the mechanisms by which pancreatic elastase induces hepatic cytokine production and the subsequent liver injury.

METHODS

Animals were cared for in accordance with the guidelines of the Department of Laboratory Animal

Presented in part at the Annual Meeting of the Association of VA Surgeons, Atlanta, Georgia, May 5–7, 2001; the Annual Meeting of the Japan Surgical Society, Sendai, Japan, April 7–14, 2001; and the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (poster presentation); and published as abstracts in *Gastroenterology* 120:A174 and A348, 2001.

From the Departments of Surgery (M.M.M., J.Y., A.F., P.K.) and Laboratory Medicine (S.M., J.G.N.), James A. Haley Veterans Hospital, University of South Florida, Tampa, Florida.

*Recipient of the 2001 SSAT Career Development Reward at the Forty-Second Annual Meeting of The Society for the Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001.

Supported by a Veterans Administration Merit Award (J.N.) and a University of South Florida–Surgery Seed Grant (M.M.).

Reprint requests: Michel M. Murr, M.D., Tampa General Hospital, P.O. Box 1289, Tampa, FL 33601. e-mail: mmurr@hsc.usf.edu

Published by Elsevier Science Inc.

1091-255X/02/$—see front matter
PII: S1091-255X(01)00071-3

Medicine at the University of South Florida, a facility accredited by the American Association for Accreditation of Laboratory Animal Care. Studies were conducted after approval was secured from our institutional review board.

In Situ Liver Perfusion (Tumor Necrosis Factor and Liver Parenchymal Enzymes)

Male Sprague-Dawely rats (250 to 350 g) were anesthetized (Nembutal, 50 mg/kg intraperitoneally). The portal vein and the suprahepatic inferior vena cava were cannulated, and the inferior vena cava was ligated to close the circuit, as described by Hems et al.[9] The liver was left in situ and perfused with oxygenated (95% O_2-5%CO_2) Krebs-Henseleit-bicarbonate (KHB) buffer at 3 to 4 ml/g until the effluent was clear. Perfusion pressure (<20 cm H_2O) and perfusate temperature (37° C) were kept constant by a heater pump. The entire perfusion system consisted of a reservoir, pump (Brinkmann Instruments, Inc., Roxbury, N.Y.), bubble trap, filter, and oxygenator. The O_2 content of the perfusate was maintained at 19 to 20 mg/ml to ensure tissue viability. The O_2 content of the effluent was 1.4 ± 0.5 mg/ml at onset of perfusion and 1.9 ± 0.6 mg/ml at the end of the experiment, indicating uniform extraction of O_2 and hence organ viability.

Subsequently the livers were perfused for 60 minutes as follows: (1) control: KHB buffer, n = 5; (2) lipopolysaccharide (LPS; 20 mg/ml) as a positive control, n = 5; and (3) elastase (1 U/ml), n = 5. The doses of LPS and elastase were based on dose-response experiments done on human monocytes and rat macrophage cell lines; the optimal dosage was determined to be the lowest dose that resulted in a maximal amount of tumor necrosis factor (TNF) response (enzyme-linked immunosorbent assay [ELISA] and reverse transcription–polymerase chain reaction [RT-PCR]) and did not affect cell viability (monotetrazolium cell proliferation assay) (unpublished data).

To determine the role of Kupffer cells in hepatic cytokine production, rats (n = 5) were pretreated with gadolinium (Gd) chloride, a well-known inhibitor of Kupffer cell function[6–8] (Gd: 10 mg/kg/day intravenously) for 48 hours, then perfused in situ with elastase (1 U/ml). Appropriate vehicle control testing was done for all groups (n = 10).

The effluent from the liver was collected every 15 minutes and stored at −80° C. LDH, AST, and ALT were determined using a Kodak Ektachem 700 automated analyzer standardized for murine proteins (Kodak, Rochester, New York). TNF protein was measured by using a commercially available rat ELISA kit (BioSource International, Camarillo, California).

In Situ Liver Perfusion (Lethal Hepatic Injury and Hepatocyte Death)

In another set of experiments, rats were randomized to receive Gd (10 mg/kg/day intravenously, n = 5) or vehicle (saline solution, n = 10) every 24 hours for 48 hours. The livers from each of these three groups were then perfused in situ with elastase (1 U/ml) with 1 mmol/L propidium iodide-KHB buffer (n = 10) or KHB buffer alone (control, n = 5) for 60 minutes. Subsequently the livers were flushed with KHB buffer to remove the excess stain and fixed by flushing 50 ml of 4% ice-cold formalin through the portal vein. Fixed tissue was embedded in paraffin and examined with a fluorescent microscope (Nikon Optiphoto-2, Tokyo, Japan; 490 nm excitation and 520 nm emission). Slides were blinded, and nonviable cells were counted per 10 high-power fields in 10 random sections of each liver.

Kupffer Cell Tissue Cultures

Freshly isolated rat Kupffer cells were provided by Dr. Hide Tsukamoto of the Non-Parenchymal Liver Cell Isolation Core at the University of Southern California Research Center for Liver Disease and the USC-UCLA Research Center for Alcoholic Liver and Pancreatic Diseases. Briefly, cells were isolated from male Wistar rats by in situ sequential digestion of the liver with pronase and collagenase, low-speed centrifugation to remove parenchymal cells, and subsequent separation of a Kupffer cell–enriched fraction by discontinuous arabinogalactin gradient centrifugation.[10]

Cells were incubated in Dulbecco minimum essential medium–5% fetal calf serum medium for 24 hours before any treatment was begun and nonadherent cells were removed. Tissue cultures of pure Kupffer cells (>98%) were plated in 24-well plates (2.5×10^6 cells/well), and pretreated with Gd (0.5 mg/ml) for 24 hours or pyrrolidine dithiocarbamate (PDTC; 0. 5 mg/ml, which inhibits TNF production via NF-κB) for 1 hour. The supernate was collected from each 2 hours after treatment with elastase (1 U/ml), and ELISA was used to determine TNF protein.

In a separate experiment, 10^7 cells/well were seeded in 100 ml dishes and treated with elastase (1 U/ml); TNF-mRNA (RT-PCR) and NF-κB activation (EMSA) were determined. Kupffer cell viability was assessed by exclusion of trypan blue.

Kupffer Cell TNF mRNA

TNF mRNA was measured by semiquantitative differential RT-PCR, as previously outlined.[5] Briefly, total Kupffer cell RNA was isolated by guanidium thiocyanate/acid phenol extraction and primed using oligo(dT) (Gibco, Gaithersburg, Md.) and subsequently reversed transcribed with reverse transcriptase (Superscript II, Gibco). The cDNA products were coamplified in the presence of murine-specific TNF and BMG primers for 20 to 25 cycles of PCR in a UNO-Thermoblock (Biometra, Tampa, Florida). The sequence for the TNF primer was sense 5′ATGAGCACAGAAAGCATGATC3′ and antisense 5′TACAGGCTTGTCACTCGAATT3′. The BMG primer sequence was sense 5′CTCCCCAAATTCAAGTGTACTCTCG3′ and antisense 5′GAGTGACGTGTTTAACTCTGCAAGC3′ (Ransom Hill Biosciences, Ramona, California). All primers are known to span at least one intron. The reaction products were separated with electrophoresis in 2.5% metaphor gel agarose containing ethidium bromide and photographed digitally under ultraviolet light with the UV Gel Documentation System (UVP, Upland, California). Band intensity of each sample was determined using GDS image analysis software (UVP), and individual cytokine/B-actin cDNA ratios were calculated for analysis.

Determination of NF-κB Activation by Electrophoretic Mobility Shift Assay

NF-κB–specific consensus oligonucleotide (5′AGTTGAGGGTTTCCCAGGC3′, Promega Corp., Madison, Wis.) was 5′ end-labeled with γ^{32}P adenosine triphosphate (ICN, Costa Mesa, California) using polynucleotide kinase (Gibco). Samples containing 10 μg of nuclear protein extract were incubated in binding buffer (10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, 1 mmol/L EDTA, 4% glycerol, and 80 μg/ml sonicated sperm DNA) with or without excess unlabeled NF-κB–specific oligonucleotide for 15 minutes on ice. End-labeled NF-κB (1.5 × 103 cycles/min) was added, and samples were incubated for an additional 45 minutes at room temperature. Free oligonucleotide and oligonucleotide-bound protein were separated by electrophoresis on a native 6% polyacrylamide gel. Gels were dried under vacuum on Whitman paper and exposed to Kodak BioMax MS film for 3 to 6 hours at −80° C. Absence of binding in the presence of excess unlabeled NF-κB–specific oligonucleotide confirmed NF-κB–binding specificity.

Hepatocyte Tissue Cultures

Hepatocytes were isolated from male Sprague-Dawley rats (300 to 350 g) by digestion with collagenase.[11] Livers were perfused in situ through the portal vein until cleared of blood with 10 mmol/L HEPES-buffered saline solution (0.15 mol/L NaCl, 0.42 g/L KCl, 0.99 g/L glucose, 2.1 g/L $NaCO_3$, and 0.19 g/L EDTA), and then perfused for 4 to 7 minutes with modified HEPES-buffered saline (no EDTA, 3.5mmol/L $CaCl_2$, 1% bovine serum albumin, and 0.025% collagenase). The liver parenchyma was dispersed manually in HEPES buffer and filtered through a stainless steel mesh (200 nm) and a Nytex mesh (75 nm), respectively. Dispersed cells were collected by centrifugation for 3 minutes at 50 g and washed three times with HEPES buffer. Cells were then resuspended and plated in modified Eagle minimum essential medium containing 10% fetal calf serum, 2.4 mmol/L glutamine, penicillin (100 units/ml), gentamicin (100 μg/ml), porcine insulin (10 μg/ml), 0.2% bovine serum albumin, and epidermal growth factor (EGF; 1 μg/100ml, Becton Dickinson Labware, Bedford, Massachusetts). Cells were plated at a density of approximately 2×10^6 in 24-well primary tissue culture plates (Becton Dickinson Labware, Franklin Lakes, New Jersey) and kept at 37° C in humidified air with 5% CO_2. The medium was replaced and nonadherent cells were removed in preparation for treatment.

Hepatocyte cultures were treated with pancreatic elastase (1 U/ml) or rat recombinant TNF (20 ng/ml). LDH, AST, and ALT, and were measured 4 hours after treatment.

Chemicals

Gadolinium chloride ($GdCl_3.6H_2O$), pancreatic elastase, and PDTC were purchased from Sigma (St. Louis, Missouri).

Statistical Analysis

Experiments were repeated in triplicate (not gels) and averaged. Data are mean ± SEM. Student's *t* test was used. Significance was set at $P < 0.05$.

RESULTS

In Situ Liver Perfusion (TNF and Liver Parenchymal Enzymes)

Elastase induced dramatic TNF protein production (Fig. 1; $^*P < 0.01$ vs. sham) and release of parenchymal enzymes into the effluent as compared to control (increased AST, ALT, and LDH, Fig. 2; $^*P < 0.03$). Interestingly, the magnitude of TNF production induced by elastase was similar to that induced by near-lethal doses of LPS (see Fig. 1). Pretreatment with Gd blunted the effect of elastase on TNF

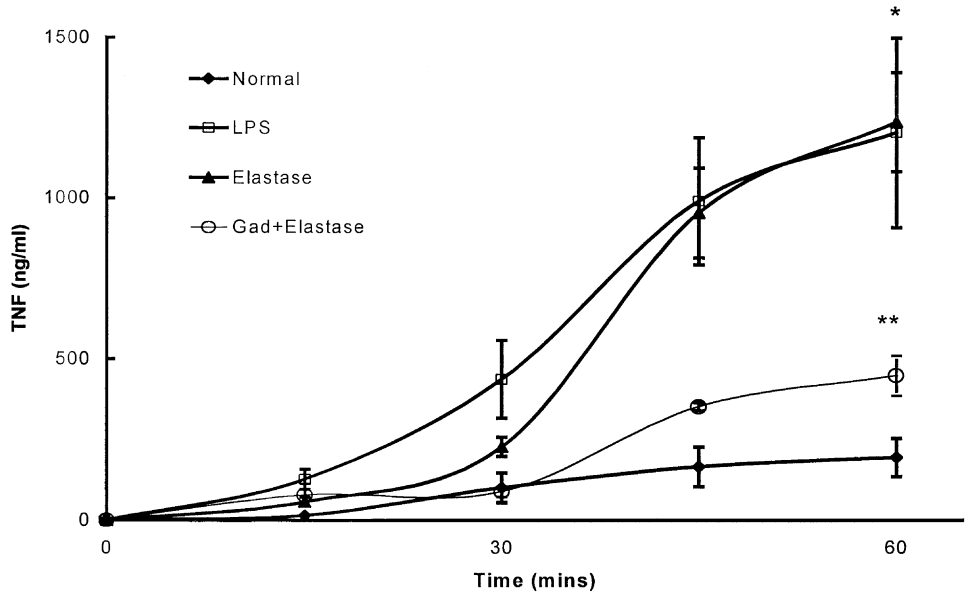


Fig. 1. Elastase-induced TNF production as compared to sham control (*$P < 0.03$; elastase vs. control). The levels of TNF induced by elastase were similar to sublethal doses of LPS. Pretreatment with Gd significantly attenuated TNF production as compared to elastase alone (**$P < 0.03$ Gd plus elastase vs. elastase alone).

production (see Fig. 1; **$P < 0.03$) and all parenchymal enzymes (see Fig. 2; **$P < 0.03$).

In Situ Liver Perfusion (Lethal Hepatic Injury and Hepatocyte Death)

Perfusion with elastase increased the number of nonviable cells that stained with propidium iodide as compared to sham controls (220 ± 26 vs. 45 ± 20, elastase vs. sham; $P < 0.001$). Pretreatment of the rats with Gd significantly blunted the effect of elastase, as evidenced by the decrease in the number of nonviable cells (71 ± 11 vs. 220 ± 26, elastase plus Gd vs. elastase alone; $P < 0.001$).

Kupffer Cell and Hepatocyte Tissue Cultures (TNF Production)

Elastase induced TNF production from tissue cultures of Kupffer cells (69 ± 22 pg/ml vs. 20 ± 2 pg/ml, elastase vs. control; $P < 0.0001$) but not from hepatocytes (24 ± 3 pg/ml). Moreover, TNF but not elastase induced an increase in the culture medium levels of AST, ALT, and LDH from primary tissue cultures of rat hepatocytes (AST: 1175 ± 80 vs. 322 ± 22 U/L; ALT: 376 ± 80 vs. 256 ± 4 U/L; LDH: 5140 ± 76 vs. 4220 ± 78 U/L; TNF vs. control; all $P \leq 0.003$). Elastase had no appreciable direct effect on liver parenchymal enzymes as compared to control values ($P > 0.05$).

Kupffer Cell Tissue Cultures (Inhibition of TNF Production)

Gd significantly attenuated the elastase-induced TNF production by Kupffer cell tissue cultures in a dose-dependent manner (Gd: 0.25 to 1.0 mg/ml) without affecting the viability of Kupffer cells (data not shown, $P < 0.001$ all doses). At a low and effective dose of 0.5 mg/ml, Gd inhibited TNF production from Kupffer cells that were treated with elastase and diminished it almost to control levels (Fig. 3; *$P < 0.0001$). Similarly, PDTC, which inhibits activation of NF-κB, abolished elastase-induced TNF production from Kupffer cells (see Fig. 3; **$P < 0.0001$).

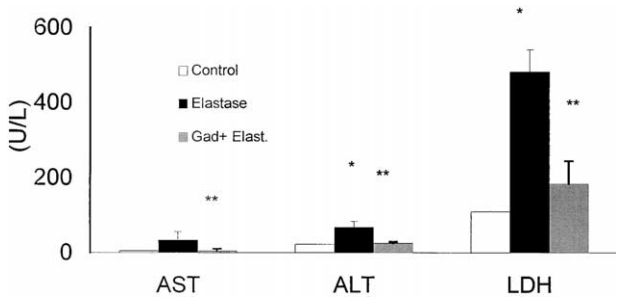


Fig. 2. The elastase-induced parenchymal liver injury (increased AST, ALT, and LDH) was attenuated by Gd in an in situ liver perfusion model (*$P < 0.03$ elastase vs. control; **$P < 0.03$ Gd plus elastase vs. elastase alone).

Kupffer Cell Tissue Cultures (TNF Gene Expression)

Elastase induced significant upregulation of TNF mRNA and activation of NF-κB in Kupffer cells within 2 hours of treatment (Fig. 4); TNF/BMG (beta-2 microglobulin) ratio was: 0.301 ± 0.031 vs. 0.022 ± 0.014; elastase vs. control ($P < 0.0001$). Pretreatment of Kupffer cell cultures with Gd inhibited the elastase-induced upregulation of TNF mRNA and attenuated the activation of NF-κB. TNF/BMG ratio was: 0.038 ± 0.018 vs. 0.301 ± 0.031, Gd plus elastase vs. elastase alone ($P < 0.0001$).

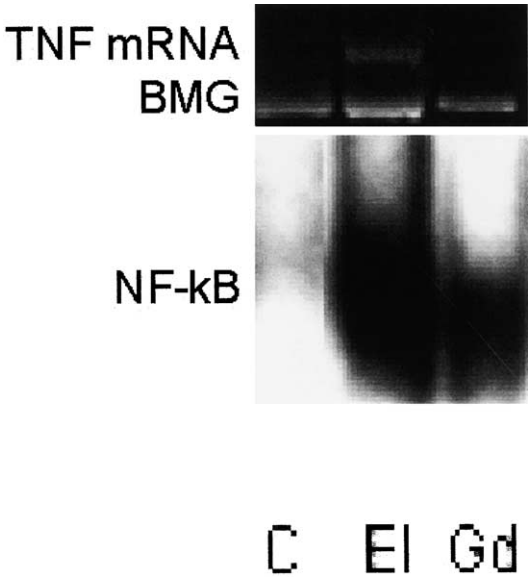


Fig. 4. Elastase (*El*)–induced upregulation of TNF mRNA and activation of NF-κB within Kupffer cells as compared to control (*C*). Pretreatment with Gd inhibited both elastase-induced upregulation of TNF mRNA and activation of NF-κB.

DISCUSSION

The role of macrophage-derived cytokines in the pathogenesis of acute pancreatitis is well established;[1] however, the mechanisms by which cytokines induce distant organ injury in severe acute pancreatitis are not well understood. The liver is a unique organ because it houses the largest population of fixed tissue macrophages and because liver injury is an important clinical prognostic indicator in severe acute pancreatitis. We have postulated that resident macrophages induce parenchymal injury in the lung and liver in experimental models of severe acute pancreatitis.[4,5,12] However, those whole-animal models introduced certain confounding factors such as mononuclear infiltration of the target organs, pancreatic ascites, and concomitant systemic severe inflammation.[13] To eliminate these confounding factors, we investigated TNF production and parenchymal liver injury in an established in situ liver perfusion model. In this model the morphologic-humoral milieu of parenchymal and nonparenchymal liver cells was preserved. Therefore any cytokine production in this setting is primarily hepatic in origin, especially if the liver is devoid of circulating blood cells.

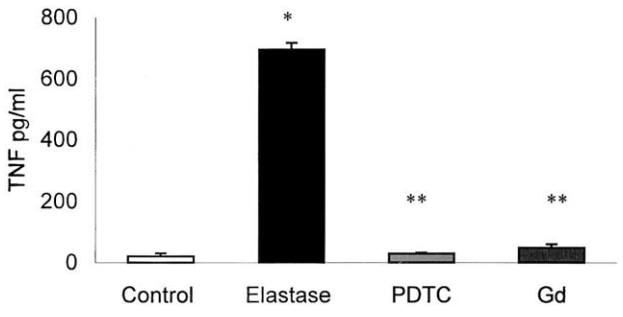


Fig. 3. Elastase induced massive production of TNF from tissue cultures of Kupffer cells as compared to control (*$P < 0.0001$ elastase vs. control). PDTC (0.5 mg/ml) or Gd (0.5mg/ml) significantly attenuated the elastase-induced TNF production within Kupffer cells (**$P < 0.0001$ PDTC or Gd vs. elastase).

We used elastase as a surrogate for acute pancreatitis because we have found that it mimics the effects of acute pancreatitis in various models in our laboratory. Elastase induced lung injury and upregulation of inflammatory mediators that are indistinguishable from diet-induced pancreatitis in laboratory animals.[4] Similarly, exposure of mice livers to elastase induced liver inflammation and upregulated TNF mRNA in an identical manner to that seen in diet-induced pancreatitis.[5] The use of elastase to induce and study the systemic effects of pancreatitis is best suited for our liver perfusion model. It eliminates the variability in severity of laboratory-induced acute pancreatitis and, more important, the local inflammatory process in the pancreas and its associated hypovolemia, which may potentiate existing liver injury. We used elastase in doses similar to those attained in the serum during severe acute pancreatitis, which have been validated to activate cytokine release from macrophages and human monocytes.[14]

In these experiments, pancreatic elastase produced a significant increase in liver parenchymal enzymes similar to that seen during severe acute pancreatitis. In addition, elastase induced intrahepatic de novo synthesis and release of TNF that reached a magnitude similar to that induced by sublethal doses of LPS. Moreover, elastase significantly increased the

number of nonviable cells, as measured by propidium iodide staining, in the perfused liver as compared to control specimens. Pretreatment with Gd, which is a well-known inhibitor of Kupffer cells,[6–8] significantly inhibited TNF production and attenuated all parameters of biochemical and histomorphologic liver injury, suggesting that elastase-induced TNF production and liver injury is mediated by Kupffer cells.

Based on observations in the liver perfusion model, we subsequently used tissue cultures to determine the source of cytokine production and to examine TNF gene expression within hepatocytes or Kupffer cells. As expected, elastase induced TNF protein production in cultures of Kupffer cells but not from hepatocytes. Moreover, TNF but not elastase induced hepatocyte injury, as evidenced by a significant increase in levels of liver parenchymal enzymes in the culture medium. These data indicate that Kupffer cells and not hepatocytes are responsible for the elastase-induced TNF production and that hepatocyte injury is caused by Kupffer cell–derived TNF and not as a direct effect of elastase.

To assess TNF gene expression and one potential signal transduction pathway involving NF-κB, we incubated Kupffer cells with either PDTC, which inhibits the activation of NF-κB, or Gd, which is known to block Kupffer cell function.[6–8] Elastase induced upregulation of TNF mRNA and activation of NF-κB. As expected, PDTC inhibited TNF production, thereby confirming that NF-κB plays an important role in elastase-induced TNF gene expression within Kupffer cells. Similarly, Gd inhibited TNF production from Kupffer cells by downregulating TNF mRNA and attenuating the activation of NF-κB. These findings are in agreement with our previous observations of the pivotal role of NF-κB in activation of TNF production from enzymatically activated macrophages.[14]

NF-κB is a family of gene transcription regulators that exist in an inactive form in the cytoplasm bound to an inhibitor (I)-κB. Phosphorylation of I-κB results in its dissociation from NF-κB, which is then translocated to the nucleus. The central role of NF-κB in the stress response and as a proximal cytokine gene regulator is well established.[15] Therefore characterization of the role of NF-κB in TNF gene expression in severe acute pancreatitis is crucial to our attempts to manipulate expression of the latter and the subsequent organ injury.

Lessons learned from clinical trials and laboratory data indicate that cytokine antagonism reduces the mortality of severe acute pancreatitis but does not abolish it.[1–3] We believe that blockade of pivotal and proximal steps in signal transduction rather than generic blockade of circulating cytokines may offer a possibility of sustainable attenuation of cytokine production and the subsequent organ injury.

In search of specific inhibitors of Kupffer cell function that will allow us to manipulate their TNF production, we used Gd because of the literature regarding its effect on Kupffer cell function during sepsis and shock.[6–9] We made a novel observation regarding a potential mechanism by which Gd inhibits Kupffer cell function; Gd attenuated elastase-induced TNF gene expression by inhibiting activation of NF-κB without affecting the viability of Kupffer cells. This is an important observation because the mechanism by which Gd exhibits its effect is controversial. Many have suggested that Gd impairs Kupffer cell phagocytosis,[16] blocks calcium channels,[17] and induces apoptosis in alveolar macrophages.[18] Our findings are complementary and novel because we are reporting a new mechanism by which Gd inhibits TNF gene expression by modulating proximal and pivotal events in the cytokine cascade. Nevertheless, further investigations are needed to elucidate the role of Gd and its effect on protein kinases and other signal transduction pathways.

We thank Martha Entel for her administrative help in preparing the manuscript and Dr. Hide Tsukamoto for providing Kupffer cells and technical advice.

REFERENCES

1. Norman J. Role of cytokines in the pathogenesis of acute pancreatitis. Am J Surg 1998;175:76–83.
2. Norman J, Fink G, Franz M. Acute pancreatitis induces intrapancreatic tumor necrosis factor gene expression. Arch Surg 1995;130:966–970.
3. Norman J, Franz M, Riker A, Gower W. Rapid elevation of cytokines during acute pancreatitis and their origination within the pancreas. Surg Forum 1994;45:148–150.
4. Yang J, Jaffray C, Carter G, Norman J. Pancreatic elastase-induced lung injury mimics pancreatitis-associated ARDS. Dig Dis Sci (submitted for publication).
5. Jaffray C, Yang J, Norman J. Elastase mimics pancreatitis-induced hepatic injury via inflammatory mediators. J Surg Res 2000;90:95–101.
6. Gloor B, Todd K, Lane J, Lewis M, Reber H. Hepatic Kupffer cell blockade reduces mortality of acute hemorrhagic pancreatitis in mice. J GASTROINTEST SURG 1998;2:430–435.
7. Zhu X, Zellweger R, Ayala A, Chaudry I. Cytokine gene expression in Kupffer cells following hemorrhage. Cytokine 1996;8:134–140.
8. Koo D, Chaudry I, Wang P. Kuppfer cells are responsible for producing inflammatory cytokines and hepatocellular dysfunction during early sepsis. J Surg Res 1999;83:151–157.
9. Hems R, Ross BD, Berry MN, Krebs HA. Gluconeogenesis in the perfused rat liver. Biochem J 1966;101:284–292.
10. Kamimura S, Tsukamoto H. Cytokine gene expression by

Kupffer cells in experimental alcoholic liver disease. Hepatology 1995;21:1304–1309.
11. Lee SJ, Friedman SL, Whalen R, Boyer TD. Cellular sources of glutathione S-transferase P in primary cultured rat hepatocytes: Localization by in situ hybridization. Biochem J 1994;229:79–83.
12. Yang J, Denham W, Carter G, Tracey KJ, Norman J. Macrophage pacification reduces pancreatitis-induced hepatocellular injury through downregulation of hepatic TNF and IL-1. Hepatology 1998;28:1282–1288.
13. Denham W, Yang J, Fink G, Zervos EE, Carter G, Norman J. Pancreatic ascites as a powerful inducer of inflammatory cytokines: The role of known vs. unknown factors. Arch Surg 1997;132:1231–1236.
14. Jaffray C, Mendez C, Denham W, Carter G, Norman J. Specific pancreatic enzymes activate macrophages to produce TNF-alpha: Role of Nuclear Factor Kappa B and Inhibitory Kappa B proteins. J GASTROINTEST SURG 2000;4: 370–378.
15. Pahl HL. Activation and target genes of Rel/NF-κB transcription factors. Oncogene 1999;18:8653–8666.
16. Husztik E, Lazar G, Parducz A. Electron microscope study of Kupffer cell phagocytosis blockade induced by gadolinium chloride. Br J Exp Pathol 1980;61:624–630.
17. Roland C, Naziruddin B, Mohanakumar T, Flye W. Gadolinium blocks rat Kupffer cell calcium channels: Relevance to calcium-dependent prostaglandin E_2 synthesis and septic mortality. Hepatology 1999,29:756–765.
18. Mizgerd J, Molina R, Stearns R, Brain J, Warner A. Gadolinium induces macrophage apoptosis. J Leukocyte Biol 1996;59:189–195.

Reduction in Mortality With Delayed Surgical Therapy of Severe Pancreatitis

Werner Hartwig, M.D., Sasa-Marcel Maksan, M.D., Thomas Foitzik, M.D., Jan Schmidt, M.D., Christian Herfarth, M.D., Ernst Klar, M.D.

The indications for surgery in acute pancreatitis have changed significantly in the past two decades. Medical charts of patients with acute pancreatitis treated at our institution were analyzed to assess the effects of changes in surgical treatment on patient outcomes. A total of 136 patients with radiologically defined severe pancreatitis were primarily treated or referred to our institution between 1980 and 1997. Severity of the disease (Ranson score), indications for surgical intervention, timing of surgery, and mortality rates were compared during three study periods: 1980 to 1985 (period I), 1986 to 1990 (period II), and 1991 to 1997 (period III). In period I patients underwent exploratory laparotomy if their clinical status did not improve markedly within 72 hours of admission to the hospital, whereas during period II surgery was reserved for patients who had secondary organ failure together with pancreatic necrosis seen on CT scan. During period III the aim was to operate as late as possible in the presence of pancreatic necrosis or when infected necrosis was suspected. The policy of limiting the indications for surgery resulted in a decrease in surgically treated patients from 68% to 33% ($P < 0.001$). Likewise, surgical intervention was performed later. In period I, 73% of operations were performed within 72 hours of admission, compared to 32% in period III ($P = 0.008$). The mortality rate for patients who underwent early surgery (within 72 hours) was higher than for those who underwent late surgical exploration of the abdomen ($P = 0.02$). Overall, the mortality rate for patients with severe pancreatitis was reduced from 39% to 12% ($P = 0.003$). Mortality among patients treated nonoperatively did not change significantly. The present study supports the policy of delayed surgery in severe acute pancreatitis. Early surgical intervention often results in unnecessary procedures with an increase in the number of deaths. Whenever possible, prolonged observation allows selection of patients who are likely to benefit from delayed surgery or nonoperative treatment. (J GASTROINTEST SURG 2002;6:481–487.)

KEY WORDS: Necrotizing pancreatitis, surgery, clinical

Three decades ago, the mortality rate for patients with severe pancreatitis was often higher than 50%, but because of improved diagnostic methods and treatment modalities the mortality rate has been lowered to 10% to 20%.[1–6] Changes in surgical procedures and, in particular, limiting the indications for surgical intervention has contributed significantly to the reduced mortality rates in severe pancreatitis.[3,7,8] However, the indications for surgery and the optimal timing of the operation are still controversial. The present study analyzed patients with severe pancreatitis treated at our institution between 1980 and 1997, focusing on a changing policy regarding the indications for surgery and the timing of surgical intervention in relation to patient mortality.

MATERIAL AND METHODS

A total of 518 consecutive patients with a first attack of acute pancreatitis were treated primarily or referred to our institution between 1980 and 1997. The diagnosis of acute pancreatitis was based on the presence of upper abdominal pain in conjunction with serum amylase levels three times normal. Se-

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (poster presentation).

From the Department of General Surgery (W.H., S.-M.M., T.F., J.S., C.H., E.K.), University of Heidelberg, Heidelberg, Germany.
Reprint requests: Ernst Klar, M.D., Chirurgische Universitätsklinik, Im Neuenheimer Feld 110, 69120 Heidelberg, Germany. e-mail: ernst_klar@med.uni-heidelberg.de

1091-255X/02/$—see front matter
PII: S1091-255X(02)00008-2

vere pancreatitis was present in 136 of these patients. Severe pancreatitis was radiologically defined as multiple peripancreatic fluid collections on conventional or contrast-enhanced CT (Balthazar grade E)[9] and/or perfusion deficit in pancreatic tissue. Contrast-enhanced CT is widely accepted to be the "gold standard" for discriminating mild edematous from severe necrotizing pancreatitis.[10] Also, patients with inconclusive CT scans and pancreatic necrosis confirmed at surgical exploration of the abdomen were included. Patients with biliary pancreatitis who had been treated endoscopically (available during the period 1986 to 1997) and patients with acute pancreatitis after cardiac surgery were excluded.

There have been three periods during which the indications for surgery have changed significantly. In period I (1980 to 1985), patients underwent exploratory laparotomy if their clinical status did not markedly improve within 72 hours of admission. During period II (1986 to 1990), surgery was reserved for patients who had secondary organ failure in association with pancreatic necrosis seen on CT scans. In period III (1991 to 1997), the aim was to operate "as late as possible" in the presence of sterile pancreatic necrosis or when infected necrosis was suspected. Surgical intervention was only performed in patients with pancreatic necrosis on CT scan and rapidly progressive multiple organ failure who did not respond to intensive care treatment, in patients with multiple organ failure and suspected infected necrosis (necrosis on CT scan combined with a rectal temperature $>38.5°$ C, blood leukocyte level <4.0 or $>12.0 \times 109$/L, and a positive blood culture), or in patients with persisting organ complications and infected necrosis proved by percutaneous fine-needle aspiration. Patients who underwent exploratory laparotomy in situations where the CT scan was inconclusive and where pancreatic necrosis was confirmed intraoperatively were included in all three periods. Patients who underwent surgery at referring hospitals were not included in the analysis. Medical charts of patients were analyzed for Ranson scores,[11] organ failure, indications for surgical intervention, timing of the operation, and mortality.

RESULTS

There were 38 patients in period I (1980 to 1985), 40 patients in period II (1986 to 1990), and 58 patients in period III (1991 to 1997). The severity of the pancreatitis, as determined by the Ranson score,[11] and the percentage of patients referred to our institution were comparable in the three groups (Table 1).

Table 1. Patient groups

	Period		
Group	**1980–1985**	**1986–1990**	**1991–1997**
Patients	38	40	58
Patients referred	11% (4/38)	13% (5/40)	21% (12/58)
Ranson-Score points	5.0 ± 0.2	4.7 ± 0.2	4.6 ± 0.3

No significant differences in Ranson Score points were found between the three time periods. Ranson scores were calculated from charts of the hospital where patients have initially been admitted. The percentage of patients referred to our institution did not change significantly.

Sixty-eight percent (26/38) of patients with severe pancreatitis were treated surgically during period I. In periods II and III, 42% (17/40) and 33% (19/58) of these patients, respectively, underwent surgery (Fig. 1). Therefore the incidence of surgery was significantly reduced (period I vs. period II, $P = 0.03$; period I vs. period III, $P < 0.001$).

At the same time, surgery was performed later in periods II and III compared to period I (Fig. 1). Seventy-three percent (19/26) of surgical interventions were performed within the first 72 hours of admission in period I, whereas only 29% (5/17) of operations in period II and 32% (6/19) of operations in period III were performed within the first 72 hours (period I vs. period II, $P = 0.01$; period I vs. period III, $P = 0.008$). The mortality rate was higher for patients who were treated surgically within the first 72 hours compared to those operated on after 72 hours (53% vs. 22%, respectively; $P = 0.02$; Table 2). In patients who have been transferred to our institution, the time point for surgery has always been calculated from primary admission to the initial hospital.

Reflecting the changed indications for surgery, significantly more patients presented with multiple organ failure at the time of surgery in period III compared to period I (79% vs. 23%, respectively; $P < 0.001$; Table 3). Likewise, more patients with single-organ failure underwent surgery in period I compared to period III (54% vs. 16%, respectively; $P = 0.01$). The percentage of patients in whom necrosectomy was performed showed no significant difference in the three periods (see Table 3).

By that means, overall mortality for patients with severe pancreatitis decreased from 39% in period I to 12% in period III ($P = 0.003$; see Fig. 1). The mortality rate for patients treated nonoperatively did not change significantly (see Table 2).

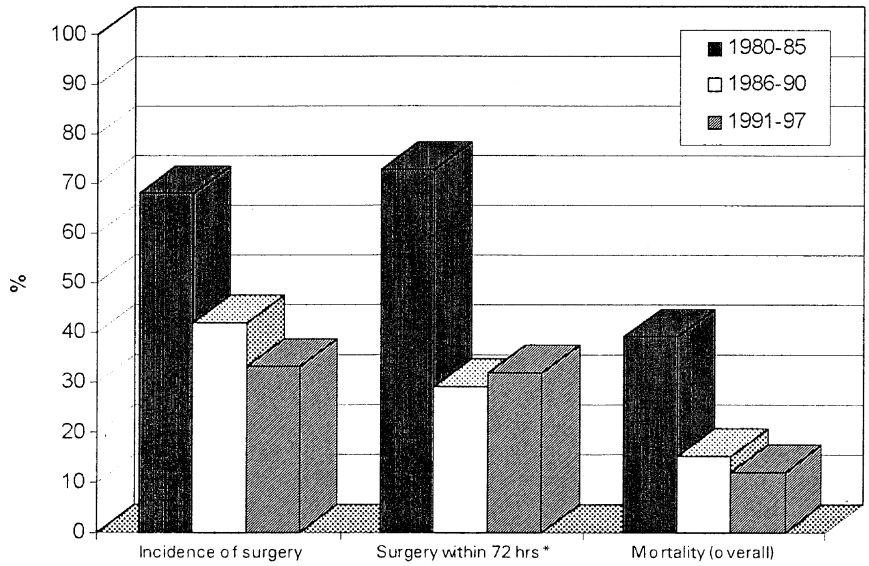


Fig. 1. Incidence of surgery, timing of surgery, and overall mortality. A significant decrease in the incidence of surgery, timing of surgery, and overall mortality was found when period I was compared with periods II and III. Timing of surgery in hours after primary admission to the initial hospital. *Percentage of patients with surgical intervention who were operated on within 72 hours.

DISCUSSION

Overall mortality for acute pancreatitis, as well as mortality for severe pancreatitis, has decreased significantly during the past few decades. Mortality rates approaching 50% in necrotizing pancreatitis were described in the 1960s, whereas at present mortality rates of 10% to 20% are achieved at specialized institutions (Fig. 2). Likewise, mortality rates for patients with severe pancreatitis treated at our institution decreased from 39% in the years 1980 to 1985 to 12% in the years 1991 to 1997.

The principal finding in this retrospective analysis is that the mortality rate for severe pancreatitis has been decreased significantly by reducing the frequency of surgical therapy and by delaying the time point of surgical intervention. Between 1980 and 1985 (period I), patients with severe pancreatitis underwent exploratory laparotomy at our institution if their clinical status did not improve markedly within 72 hours of admission. This policy was based on the optimistic results of early surgical treatment in the late 1970s.[12–15] The former rationale of removing necrotic tissue early in the course of disease was to eliminate proinflammatory mediators that are released from the damaged pancreas. Because of the high mortality rate of 39% during this period, we reevaluated this concept critically and restricted the indications for surgery between 1986 and 1990 (period II) to patients with secondary organ failure in association with pancreatic necrosis seen on CT scans. In this period the overall mortality rate decreased to 15%. During period III (1991 to 1997) the indications for surgery have been further restricted considering reports that questioned surgical intervention in the presence of sterile necrosis.[2] In addition to patients with an uncertain diagnosis, only patients with rapidly progressive multiple organ failure who did not respond to intensive care treatment or those with infected necrosis had surgery.

Table 2. Effect of the timing of surgery on mortality period

	Period			
Mortality	**1980–1985**	**1986–1990**	**1991–1997**	**Combined**
With surgery within 72 hr	58% (11/19)	40% (2/5)	50% (3/6)	53% (16/30)
With surgery later than 72 hr	43% (3/7)	17% (2/12)	15% (2/13)	22% (7/32)*
With non-operative therapy	8% (1/12)	9% (2/23)	5% (2/39)	

Mortality of patients with early surgery was higher than with late surgery. The difference was statistically significant when all three periods were combined (*$P = 0.02$). The decrease in mortality with late surgery missed statistical significance (period I vs. period III, $P = 0.29$). Timing of surgery in hours after primary admission to the initial hospital.

Table 3. Organ failure and necreosectomy in patients treated surgically

	Period		
	1980–1985	**1986–1990**	**1991–1997**
No organ failure	23% (6/26)	12% (2/17)	5% (1/19)
Single organ failure	54% (14/26)	47% (8/17)	16% (3/19)*
Multiple organ failure	23% (6/26)	41% (7/17)	79% (15/19)†
Debridement of necrosis	85% (22/26)	88% (15/17)	95% (18/19)

Reflecting the changed indication for surgery in severe pancreatitis, significantly more patients with single or no organ failure were treated surgically in period I compared to period III (*$P = 0.01$). In period III, significantly more patients presented MOV at the time of operation compared to period I (†$P < 0.001$). There were no significant differences in the percentage of patients in whom debridement of necrosis was performed.

On the basis of this policy, the mortality rate maintained low. However, in our cohort we have not been able to further decrease the mortality rate for period III as compared to the mortality for period II.

This reported policy of restricting the indications for surgery was accompanied by a decreasing incidence of surgical intervention in our patient cohort. In the three groups, which were comparable with regard to the severity of the disease, the incidence of surgical intervention declined from 68% in period I to 33% in period III. Despite narrowing the indications for surgery, the mortality rate for patients treated nonoperatively was not increased in the later periods.

The optimal timing of surgery in severe pancreatitis has been a matter of controversy. As shown by this study, the mortality rate for patients who underwent laparotomy later than 72 hours after admission was lower than for those patients treated surgically within 72 hours in all three time periods. Several factors may account for this phenomenon. It has been proposed that patients with sterile necrosis benefit

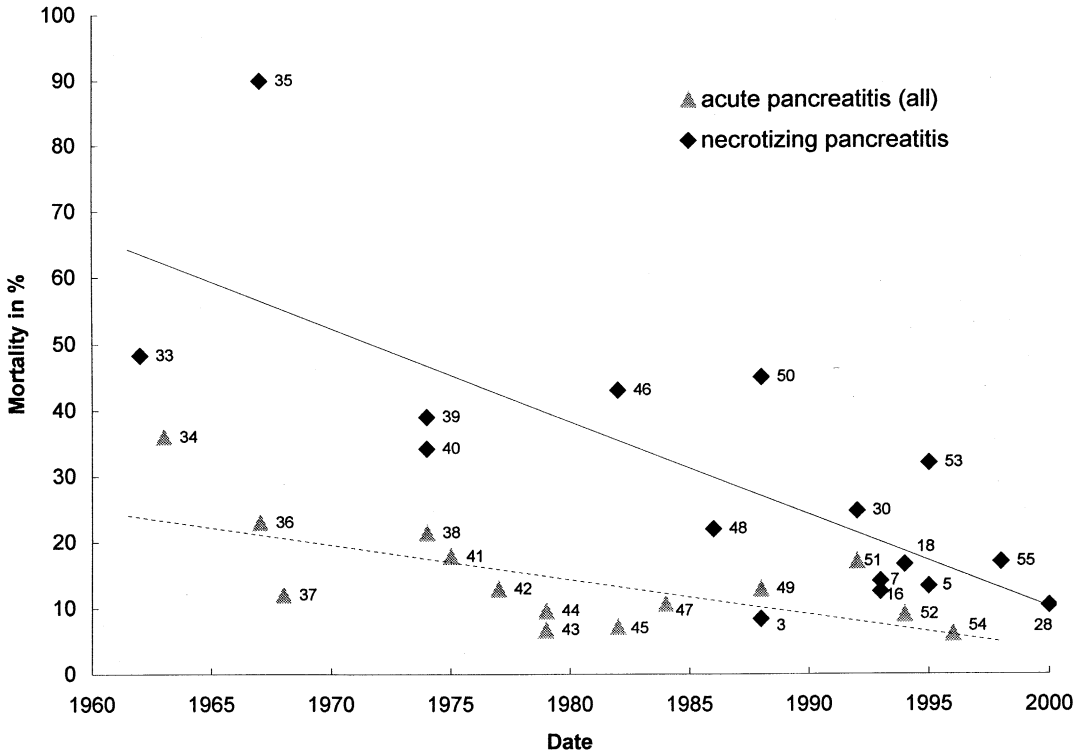


Fig. 2. Mortality of acute and necrotizing pancreatitis: Summary of previous studies. Mortality of patients with acute pancreatitis (irrespective of the severity of the disease), as well as mortality of patients with necrotizing pancreatitis, decreased significantly in the past decades. Numbers represent the studies as listed in References.

from a "wait and see" policy, even when the clinical course is complicated by organ failure.[2] The rationale for late surgery (later than 3 weeks after the onset of symptoms, when possible) is the ease of identifying well-demarcated necrotic tissue from the viable parenchyma, with the effect of limiting the extent of surgery to pure debridement.[16] The preoperative stabilization of organ dysfunction by improved treatment in the intensive care unit may be another factor. Whether patients presenting with infected necrosis shortly after admission may also benefit from delayed surgery, as suggested by some investigators,[17] remains unclear.

Unlike the rate for late surgical intervention, the mortality rate for patients who had early surgery (within 72 hours of admission) remained high throughout all observation periods, although the indications for surgery were based on different concepts. From 1980 to 1985 (period I) the aim of surgery was the early removal of the inflammatory focus. In striking contrast, from 1991 to 1997 (period III) surgery was performed within 72 hours of admission only if the patient could not be stabilized by treatment in the intensive care unit or if severe intra-abdominal disease other than pancreatitis had to be ruled out. Five of the six patients who were operated on within the first 72 hours during period III had rapidly progressive multiple organ failure that did not respond to treatment in the intensive care unit. In four of these patients, pancreatic necrosis was diagnosed as being infected intraoperatively. Two patients had extensive necrosis of the colon, which mandated resection. An inconclusive CT scan with peripancreatic fluid and suspected perforated viscus was the indication for laparotomy in another patient in whom pancreatic necrosis was confirmed intraoperatively. This subgroup of patients had extreme morbidity, thereby explaining the persistence of high mortality with early surgery.

Mortality rates up to 65% with early surgery in severe pancreatitis have also been described in others reports that questioned the benefit of surgical intervention within the first days after the onset of symptoms.[17–20] In the single prospective randomized clinical trial that compared early (within 48 to 72 hours of symptoms) and late (at least 12 days after onset) debridement in patients with severe pancreatitis, the mortality rates were 56% and 27%, respectively.[17] The high mortality rate in patients operated on early in that study was independent from the bacteriologic status of the pancreatic necrosis.

Our study confirms the current view of timing and extent of surgery in acute pancreatitis, which is based on a better understanding of the pathophysiologic mechanisms of the disease. In the early phase of pancreatitis, proinflammatory mediators are liberated from the inflamed pancreatic gland, triggering secondary organ failure.[21] However, distant organ distress can be managed by treatment in the intensive care unit, whereas pancreatic necrosis, which is not well demarcated from viable tissue in this early course of the disease, is not yet removable by blunt necrosectomy. Therefore early surgery should only be performed in patients whose conditions cannot be stabilized despite maximal treatment in the intensive care unit, or if severe pathologic conditions other than pancreatitis (e.g., perforated viscus) have to be excluded.[22–24]

When pancreatic necrosis has developed, the differentiation between sterile and infected necrosis is critical for further treatment. Proved infected necrosis, as well as septic complications resulting from pancreatic infection, are well accepted indications for surgical treatment.[3,7,24,26,28] The mortality rate in these patients is higher than 30%, and more than 80% of fatal outcomes in acute pancreatitis are due to septic complications.[20,27] Sterile necrosis, in general, is no indication for surgery, since several reports have demonstrated that patients with proved necrosis can be well managed nonoperatively.[2,28] However, when sterile necrosis is associated with organ failure, the role of surgery is still controversial.[29–31] The manifestation of single or multiple organ failure in acute pancreatitis is associated with mortality rates ranging from 23 to 75%, no matter whether pancreatic necrosis is infected or sterile.[29,30,32] Therefore a controlled study is needed to give conclusive results concerning the benefit of surgical therapy in the subset of patients presenting with sterile necrosis and secondary organ failure.

CONCLUSION

Our analysis confirms the widely accepted policy of delaying surgery in severe pancreatitis and limiting the operation to debridement. Patients benefit from preoperative clinical stabilization and demarcation of necrotic tissue. Only a few patients remain who must be treated surgically during the early course of the disease if stabilization cannot be achieved by treatment in the intensive care unit. The morbidity of these patients explains the persistence of high mortality in this subgroup.

REFERENCES

1. Banks PA, Gerzof SG, Langevin RE, et al. CT-guided aspiration of suspected pancreatic infection: Bacteriology and clinical outcome. Int J Pancreatol 1995;18:265–270.

2. Bradley EL, Allen K. A prospective longitudinal study of observation versus surgical intervention in the management of necrotizing pancreatitis. Am J Surg 1991;161:19–24.
3. Beger HG, Buchler M, Bittner R, et al. Necrosectomy and postoperative local lavage in necrotizing pancreatitis. Br J Surg 1988;75:207–212.
4. Pederzoli P, Bassi C, Vesentini S, Campedelli A. A randomized multicenter clinical trial of antibiotic prophylaxis of septic complications in acute necrotizing pancreatitis with imipenem. Surg Gynecol Obstet 1993;176:480–483.
5. Sainio V, Kemppainen E, Puolakkainen P, et al. Early antibiotic treatment in acute necrotising pancreatitis. Lancet 1995;346:663–667.
6. Warshaw AL. Damage prevention versus damage control in acute pancreatitis. Gastroenterology 1993;104:1216–1219.
7. Bradley EL. A fifteen year experience with open drainage for infected pancreatic necrosis. Surg Gynecol Obstet 1993;177:215–222.
8. Foitzik T, Klar E, Buhr HJ, Herfarth C. Improved survival in acute necrotizing pancreatitis despite limiting the indications for surgical debridement. Eur J Surg 1995;161:187–192.
9. Balthazar EJ, Ranson JH, Naidich DP, et al. Acute pancreatitis: Prognostic value of CT. Radiology 1985;156:767–772.
10. Buchler M. Objectification of the severity of acute pancreatitis. Hepatogastroenterology 1991;38:101–108.
11. Ranson JH, Rifkind KM, Roses DF, et al. Prognostic signs and the role of operative management in acute pancreatitis. Surg Gynecol Obstet 1974;139:69–81.
12. Nardi GL. Acute pancreatitis. Surg Clin North Am 1966; 46:619–626.
13. Waterman NG, Walsky R, Kasdan ML, Abrams BL. The treatment of acute hemorrhagic pancreatitis by sump drainage. Surg Gynecol Obstet 1968;126:963–971.
14. Anderson MC. Review of pancreatic disease. Surgery 1969; 66:434–449.
15. Frey CF. Hemorrhagic pancreatitis. Am J Surg 1979;137: 616–623.
16. Buchler M, Uhl W, Beger HG. Surgical strategies in acute pancreatitis. Hepatogastroenterology 1993;40:563–568.
17. Mier J, Leon EL, Castillo A, et al. Early versus late necrosectomy in severe necrotizing pancreatitis. Am J Surg 1997; 173:71–75.
18. Fernandez-Cruz L, Navarro S, Valderrama R, et al. Acute necrotizing pancreatitis: A multicenter study. Hepatogastroenterology 1994;41:185–189.
19. Kelly TR, Wagner DS. Gallstone pancreatitis: A prospective randomized trial of the timing of surgery. Surgery 1988;104:600–605.
20. Beger HG, Bittner R, Block S, Buchler M. Bacterial contamination of pancreatic necrosis. A prospective clinical study. Gastroenterology 1986;91:433–438.
21. Formela LJ, Galloway SW, Kingsnorth AN. Inflammatory mediators in acute pancreatitis. Br J Surg 1995;82:6–13.
22. Beger HG. Operative management of necrotizing pancreatitis—Necrosectomy and continuous closed postoperative lavage of the lesser sac. Hepatogastroenterology 1991;38: 129–133.
23. Poston GJ, Williamson RC. Surgical management of acute pancreatitis. Br J Surg 1990;77:5–12.
24. Bradley EL. Indications for debridement of necrotizing pancreatitis. Pancreas 1996;13:219–223.
25. Bradley EL. Management of infected pancreatic necrosis by open drainage. Ann Surg 1987;206:542–550.
26. Sarr MG, Nagorney DM, Mucha PJ, et al. Acute necrotizing pancreatitis: Management by planned, staged pancreatic necrosectomy/debridement and delayed primary wound closure over drains. Br J Surg 1991;78:576–581.
27. Bittner R, Block S, Buchler M, Beger HG. Pancreatic abscess and infected pancreatic necrosis. Different local septic complications in acute pancreatitis. Dig Dis Sci 1987;32: 1082–1087.
28. Buchler MW, Gloor B, Muller CA, et al. Acute necrotizing pancreatitis: Treatment strategy according to the status of infection. Ann Surg 2000;232:619–626.
29. Karimgani I, Porter KA, Langevin RE, Banks PA. Prognostic factors in sterile pancreatic necrosis. Gastroenterology 1992;103:1636–1640.
30. Rattner DW, Legermate DA, Lee MJ, et al. Early surgical debridement of symptomatic pancreatic necrosis is beneficial irrespective of infection. Am J Surg 1992;163:105–109.
31. Rau B, Pralle U, Uhl W, et al. Management of sterile necrosis in instances of severe acute pancreatitis. J Am Coll Surg 1995;181:279–288.
32. Tran DD, Cuesta MA, Schneider AJ, Wesdorp RI. Prevalence and prediction of multiple organ system failure and mortality in acute pancreatitis. J Crit Care 1993;8:145–153.
33. Foster PD, Ziffren SE. Severe acute pancreatitis. Arch Surg 1962;85:252–259.
34. Krupp S. Die Pankreatitis. Ätiologie, Alters- und Geschlechtsverteilung, Therapie und Mortalität an Hand von 361 Fällen. Helv Chir Acta 1963;30:367–400.
35. Nugent FW, Atendido WA, Gibb SP. Comprehensive treatment of acute hemorrhagic pancreatitis. Am J Gastroenterol 1967;47:511–517.
36. Black WS, Sutterfield TC, Martin JDJ. Acute pancreatitis. Am Surg 1967;33:94–99.
37. White TT, Murat JE, Morgan A. Pancreatitis. I. Review of 733 cases of pancreatitis from three Seattle hospitals. Northwest Med 1968;67:374–378.
38. Imrie CW. Observations on acute pancreatitis. Br J Surg 1974;61:539–544.
39. Edelmann G, Boutelier P. [Treatment of acute necrotizing pancreatitis through the early surgical ablation of necrotic portions]. Chirurgie 1974;100:155–167.
40. Warshaw AL, Imbembo AL, Civetta JM, Daggett WM. Surgical intervention in acute necrotizing pancreatitis. Am J Surg 1974;127:484–491.
41. Bourke JB. Variation in annual incidence of primary acute pancreatitis in Nottingham, 1969-74. Lancet 1975;2:967–969.
42. Jacobs ML, Daggett WM, Civette JM, et al. Acute pancreatitis: Analysis of factors influencing survival. Ann Surg 1977;185:43–51.
43. Satiani B, Stone HH. Predictability of present outcome and future recurrence in acute pancreatitis. Arch Surg 1979; 114:711–716.
44. Ong GB, Lam KH, Lam SK, et al. Acute pancreatitis in Hong Kong. Br J Surg 1979;66:398–403.
45. Ranson JH. Etiological and prognostic factors in human acute pancreatitis: A review. Am J Gastroenterol 1982;77: 633–638.
46. Cooper MJ, Williamson RC, Pollock AV. The role of peritoneal lavage in the prediction and treatment of severe acute pancreatitis. Ann R Coll Surg Engl 1982;64:42–427.
47. Blamey SL, Imrie CW, O'Neill J, et al. Prognostic factors in acute pancreatitis. Gut 1984;25:1340–1346.
48. Puolakkainen P, Lempinen M, Schroder T. Fatal pancreatitis. A study of 64 consecutive cases. Acta Chir Scand 1986; 152:379–383.
49. Wilson C, Imrie CW, Carter DC. Fatal acute pancreatitis. Gut 1988;29:782–788.
50. Teerenhovi O, Nordback I, Isolauri J. Ranson signs and ne-

crosis of the pancreas in acute necrotising pancreatitis. Acta Chir Scand 1988;154:385–388.
51. Tran DD, Cuesta MA. Evaluation of severity in patients with acute pancreatitis. Am J Gastroenterol 1992;87:604–608.
52. Mann DV, Hershman MJ, Hittinger R, Glazer G. Multicentre audit of death from acute pancreatitis. Br J Surg 1994;81:890–893.
53. Fugger R, Gotzinger P, Sautner T, et al. Necrosectomy and laparostomy—A combined therapeutic concept in acute necrotising pancreatitis. Eur J Surg 1995;161:103–107.
54. Lankisch PG, Burchard-Reckert S, Petersen M, et al. Morbidity and mortality in 602 patients with acute pancreatitis seen between the years 1980-1994. Z Gastroenterol 1996;3 4:371–377.
55. Bassi C, Falconi M, Talamini G, et al. Controlled clinical trial of pefloxacin versus imipenem in severe acute pancreatitis. Gastroenterology 1998;115:1513–1517.

Perioperative Matrix Metalloproteinase Inhibition Therapy Does Not Impair Wound or Anastomotic Healing

James H. Balcom IV, M.D., Tobias Keck, M.D., Andrew L. Warshaw, M.D., Bozena Antoniu, M.S., Gregory Y. Lauwers, M.D., Carlos Fernández-del Castillo, M.D.

Matrix metalloproteinases (MMPs) catalyze the degradation of collagen and extracellular matrix. They play a role in pathologic states including malignancy, in which they facilitate invasion and metastasis. MMP inhibition has been shown to block neoplastic invasion and improve survival in animal models of malignancy. Concern about the effects of MMP inhibitors on wound and anastomotic healing may limit their potential use in the perioperative period to prevent local and systemic showering of cancer cells from surgical manipulation. We sought to assess the safety of perioperative administration of an MMP inhibitor (BB-94) with respect to skin and bowel healing in a rat model. Absorption of BB-94 was confirmed through high-pressure liquid chromatography and mass spectroscopy of sera from treated animals. Bowel bursting pressure in all animals increased almost 10-fold between 4 and 14 days. Two-way analysis of variance showed no significant difference in bowel bursting pressure between control and treatment animals over time. There was a significant increase in the collagen content of skin specimens of all animals combined between 4 and 28 days. Similarly, all animals showed an increase in bowel collagen between 4 and 28 days. There was no significant difference in skin or bowel collagen concentrations between control and treatment animals over time. Perioperative treatment with MMP inhibition does not impair wound or enteric healing in a rat model of laparotomy and small bowel resection. MMP inhibitors are safe for use as adjuvant therapy after resection for cancer. (J GASTROINTEST SURG 2002;6:488–495.)

KEY WORDS: Matrix metalloproteinase, wound healing, anastomosis, collagen, surgical oncology

Adhesion, migration, and subsequent function of cellular elements in mammalian tissues are intimately dependent on the content and function of the extracellular matrix in which they are found. Physiologic and pathologic processes, such as wound healing and neoplastic progression, influence and are affected by changes in the extracellular matrix.[1,2] The composition of the extracellular matrix is in constant flux as collagen and other structural proteins are synthesized, deposited, and degraded.[1]

Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes that function to degrade extracellular matrix. The MMP family of enzymes includes, among others, collagenases (represented by MMP-1), gelatinases (represented by MMP-2 and MMP-9), and stromelysins (represented by MMP-3).[3] MMP activity has been described in a variety of processes including inflammation, angiogenesis, wound healing, and cancer.[2,4–6] There is now considerable evidence that MMPs may have a vital role in the malignant invasion of normal tissues by local extension and metastasis.[2,7,8] Using a nude mouse model of metastatic pancreatic ductal adenocarcinoma, we have shown that MMP inhibition with the drug BB-94 (batimastat) decreases tumor load and improves survival. Our studies also suggest that this strategy may be particularly useful in the perioperative setting of tumor extirpation, where MMP inhibition may pre-

Presented at the Sixteenth Annual SSAT/Ross Residents and Fellows Research Conference, May 19, 2001; and at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (poster presentation).
From the Departments of Surgery (J.H.B., T.K., A.L.W., B.A., C.F.C.) and Pathology (G.Y.L.), Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts.
Supported by the Edward D. Churchill, M.D. Resident Research Fellowship, Harvard Medical School, Boston, Massachusetts.
Reprint requests: Carlos Fernández-del Castillo, M.D., Massachusetts General Hospital—WACC 336, 55 Fruit St., Boston, MA 02114. e-mail: cfernandez@partners.org

Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(02)00015-X

vent showering of malignant cells into the local and systemic circulation and may prevent implantation of those same cells.[9]

The role and influence of MMPs in wound healing has been the subject of intense investigation for several years. There is evidence from numerous studies that MMPs are crucial in the coordination of wound healing and in the long-term extracellular matrix remodeling that occurs in its later stages.[1,3,4,6,10–15] Interestingly, there is evidence that MMPs and their endogenous inhibitors, tissue inhibitors of metalloproteinases, may have complex roles in wound healing, which amounts to a net increase in collagen and other extracellular matrix in some circumstances and a net decrease in matrix components in others.[1,8,12,14] Few experiments have included relevant measurements of mechanical wound strength in their assessment of the effect of MMPs on healing, particularly enteric healing. Without clear experimental evidence of an unequivocal positive or negative effect of MMPs on wound healing, the use of MMP inhibitors as perioperative adjuvants in surgical oncology has been understandably limited. For example, the ongoing clinical trial with the oral MMP inhibitor marimastat in pancreatic cancer requires a 6-week hiatus between resection and the initiation of drug treatment.[7]

The aim of this study was to determine the effect of BB-94 (batimastat), which is a broad-spectrum metalloproteinase inhibitor with proved in vitro and in vivo efficacy,[9] on wound healing in a rat model of laparotomy and small bowel resection.

MATERIAL AND METHODS

Animals, Operative Technique, and Measurement of Bowel Bursting Pressure

All animals used in these experiments were treated according to a protocol approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital in accordance with guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23, 1986). Male Sprague-Dawley rats (n = 75), weighing 150 to 250 g, were purchased from Charles River Laboratories (Wilmington, Massachusetts) and were fed standard dry chow and water ad libitum for 3 days. The animals were then fasted overnight and given only a solution of 0.225% normal saline with 5% sucrose by mouth. On the morning of operation, the animals were placed under general anesthesia with intramuscular ketamine, 44 mg/kg, and intraperitoneal pentobarbital, 20 mg/kg. Animals were then randomly assigned to one of three groups: (1) intraperitoneal BB-94, 40 mg/kg, in 20% dimethyl sulfoxide (DMSO; Sigma, St. Louis, Missouri) for 3 days beginning on the day of operation; (2) intraperitoneal BB-94, 40 mg/kg, in 20% DMSO for 14 days beginning on the day of operation; or (3) intraperitoneal solution of 20% DMSO in saline for 3 or 14 days beginning on the day of operation.

BB-94 was generously provided by British Biotech (Oxford, United Kingdom). Because of its limited water solubility, BB-94 was prepared for injection in a sterile solution of 20% DMSO in normal saline. The resulting suspension was homogeneous, and we were able to use it for injection without difficulty.

Approximately 30 minutes after the drug or carrier was injected, a midline laparotomy was performed and a loop of distal ileum elevated into the wound. A 3 cm segment of ileum was resected, and the bowel was reanastomosed in end-to-end fashion with five interrupted, inverting 5-0 polyglycolic acid sutures. The abdomen was closed in two layers with running 5-0 polyglycolic acid sutures, and the animals were allowed to recover.

The animals were given the saline and sucrose solution, which was described previously, on postoperative day 1, and thereafter they were given standard dry chow and water ad libitum. Intraperitoneal injections of drug or carrier were administered according to the schedule outlined previously, and the animals were killed by carbon dioxide inhalation on postoperative day 4, 10, 14, 28, or 49 (n = 5 animals/group). Bowel bursting pressure (BBP) was determined at that point by insufflating the anastomosed segment of bowel with air in parallel connection to a mercury sphygmomanometer column. The BBP was recorded as the pressure required to cause bowel disruption at the anastomosis. For hydroxyproline and collagen determinations, a 6 mm punch biopsy was used to obtain samples of skin from the area of the healed skin incision and intestine from the area of the bowel anastomosis. Separate specimens from both locations were (1) snap-frozen in liquid N_2 and stored at −80° C and (2) fixed in formalin for preparation of paraffin-embedded tissue blocks.

Reverse-Phase High-Pressure Liquid Chromatography and Mass Spectroscopy

Although a previous study has shown that BB-94 can be absorbed based on evidence from murine intraperitoneal injections,[16] we sought to confirm this. In four rats, 8 ml of blood was collected by cardiac puncture on day 14 of BB-94 treatment, just before the animals were killed. The separated plasma was combined into a single aliquot, and the plasma proteins were removed by methanol precipitation according to the method described by Wang, et al.[16]

Briefly, the methanol-precipitated samples were lyophilized and reconstituted in 60% acetonitrile and submitted for reverse-phase high-pressure liquid chromatography (RP-HPLC). All RP-HPLC and mass spectroscopy analyses were performed at Analytical Technology Services, Watertown, Massachusetts.

The methanol-precipitated plasma was then analyzed by means of RP-HPLC using an RP–C-8 analytical column on a Varian reverse-phase high-pressure liquid chromatograph (Varian, Inc., Palo Alto, California). The retention time of BB-94 was determined by injection of 25 μg of pure BB-94 and BB-94 "spiked" blood at different concentrations. An optimal elution gradient of 0.1% trifluoroacetic acid (TFA) and 99% acetonitrile at 1 ml/min for 20 minutes revealed desorbtion of BB-94 at 13.74 minutes. The methanol-precipitated plasma was collected in fractions every 30 seconds from 13 to 15 minutes and submitted for mass spectroscopy.

Mass spectroscopy was performed using a Voyager Elite mass spectrometer (PerSeptive Biosystems, Inc., Framingham, Massachusetts) and workstation. The 13.5-minute fraction from the methanol-precipitated plasma demonstrated a single, large peak at 478 atomic mass units (amu), which corresponds to the peak seen on mass spectroscopy of BB-94 "spiked" blood.

Hydroxyproline and Collagen Analysis

All procedures used in the determination of hydroxyproline and collagen content were based on the method described by Reddy and Enwemeka.[17] Briefly, the method involves the following three steps: (1) hydrolysis of the skin and bowel specimens; (2) oxidation of the liberated hydroxyproline; and (3) development of a chromophore with the oxidized hydroxyproline. The samples were air dried, weighed, and autoclaved at 120° C for 20 minutes in a 2 mol/L NaOH solution to liberate hydroxyproline. The samples were then mixed with 0.056 mol/L chloramine T (Sigma), which results in hydroxyproline oxidation and production of a pyrrole. Addition of 1 mol/L Ehrlich's reagent (Sigma) to the oxidized hydroxyproline results in the generation of a chromophore. The absorbance of the chromophore can be measured at 550 nm.

Known concentrations of hydroxyproline stock solutions were subjected to the process described above, and the resulting absorbance measurements were used to create a linear standard curve. Absorbance measurements from each sample, prepared as described above, were then used to obtain hydroxyproline concentrations from the standard curve. Since hydroxyproline expression is essentially limited to collagen, we assumed that collagen consisted of 12.5% hydroxyproline to calculate the collagen content,[17,18] which was normalized for tissue weight in each sample.

Trichrome Staining of Paraffin-Embedded Tissue

Slides were prepared from the previously collected paraffin-embedded skin and bowel specimens and stained with a standard Masson's trichrome staining technique. The slides were then reviewed in blinded fashion by a pathologist (G.Y.L.). A semiquantitative scoring system was used to assess the amount of collagen present in skin or bowel wounds: 0 = no organized collagen deposition in the dermis or submucosa; 1 = minimal collagen deposition; 2 = moderate collagen deposition; 3 = marked collagen deposition.

Statistical Analysis

To formulate a comprehensive interpretation of potential differences among the three experimental groups over the entire duration of the study, comparisons of the BBP values, skin collagen concentrations, bowel collagen concentrations, and histologic collagen score were conducted using a two-way analysis of variance (ANOVA) technique. Comparisons of BBP values, skin collagen concentrations, and bowel collagen concentrations at specific time points in the study were performed using Student's t test. Continuous data are presented as the mean ± SEM. Statistical significance was defined as $P < 0.05$. All statistical analyses were performed with the assistance of GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, California.

RESULTS

BB-94 treatment was well tolerated. The animals treated with BB-94 did not experience weight loss or increased mortality (data not shown).

RP-HPLC of pure BB-94 in acetonitrile and the methanol-precipitated plasma was performed as described previously. Because of the broad nature of the peak at 220 nm seen at 13.74 minutes of extraction time, multiple fractions from 13 to 15 minutes were collected during the separation (data not shown). Mass spectroscopy of BB-94 "spiked" blood and the 13.5-minute HPLC-separated fraction yielded dominant peaks clearly identified at 478 amu (data not shown); these peaks corresponded to the published molecular weight of 478 amu for BB-94.[19]

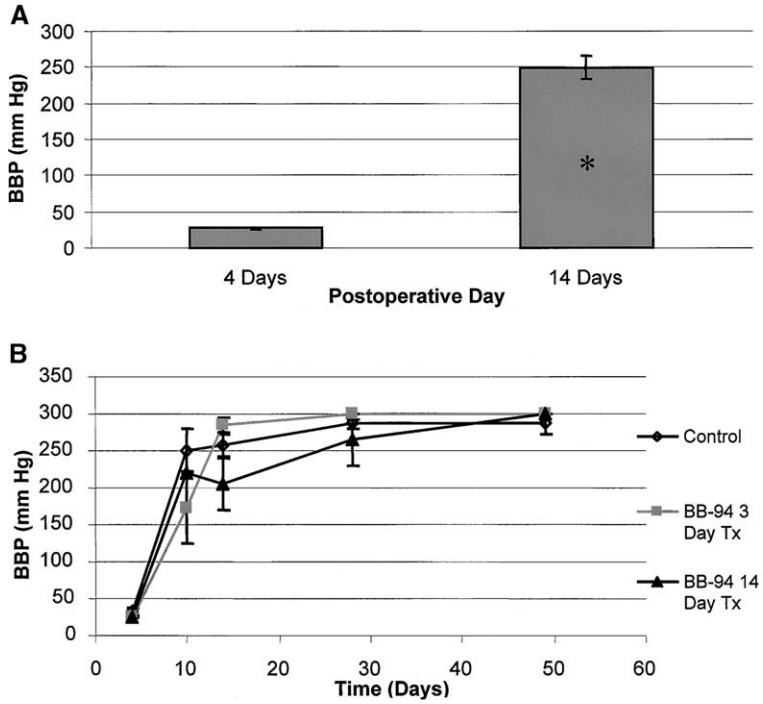


Fig. 1. A, Significant increase in BBP between 4 and 14 days for all animals killed at these time points (increase from 28 ± 2 mm Hg at 4 days to 249 ± 16 mm Hg at 14 days, $P < 0.001$; $^{*}P < 0.05$). **B**, Changes in BBP for animals in the three experimental groups over time ($P = 0.53$).

BBP values are shown in Fig. 1. Fig. 1, *A* demonstrates the near 10-fold increase in BBP between days 4 and 14 (increase from 28 ± 2 mm Hg at 4 days to 249 ± and 16 mm Hg at 14 days, $P < 0.001$). Fig. 1, *B* shows the changes in BBP for animals in the three experimental groups over time. Two-way ANOVA showed no significant differences among control animals, animals treated with BB-94 for 3 days, and animals treated with BB-94 for 14 days ($P = 0.53$).

Skin wound collagen concentrations are shown in Fig. 2. There was a significant increase in skin collagen content between 4 and 28 days for all animals combined (9.0 ± 0.8 mg/g tissue at 4 days vs. 11.7 ± 0.8 mg/g tissue at 28 days, $P < 0.05$) (Fig. 2, *A*), but there was no significant difference in skin wound collagen concentrations among the three experimental groups over time (Fig. 2, *B*; $P = 0.94$).

Fig. 3 shows the results of collagen concentration determinations in the bowel anastomoses of the experimental animals. Bowel collagen concentrations increased significantly between 4 and 28 days for all animals killed at these time points (2.0 ± 0.2 mg/g tissue at 4 days vs. 4.8 ± 0.6 mg/g tissue at 28 days, $P < 0.001$) (Fig. 3, *A*), but bowel collagen content did not change significantly over time among the three experimental groups by two-way ANOVA (Fig. 3, *B*; $P = 0.63$).

Results of histologic collagen trichrome staining of rat skin and bowel anastomoses are illustrated in Fig. 4. There were no significant differences in the histologic collagen scores in rat skin or bowel among the three experimental groups over time ($P > 0.05$ for both). Typical trichrome stain results for early and late bowel anastomoses in control and treatment animals are presented in Fig. 5. Note that the early and late scars show the presence of similar amounts of collagen in control and treatment animals.

DISCUSSION

The effects of MMP inhibition on wound healing and extracellular matrix turnover are complex and are not fully understood.[1,3,5,20] Different MMPs appear to be expressed at different time points in the healing process. For example, whereas MMP-1 and MMP-9 appear to be upregulated early in the inflammatory and epithelial migration/proliferation periods of healing, MMP-2 seems to increase and remain elevated 5 days after wounding.[1,3,20] Thus MMPs seem to have roles in both the acute phase of healing and the later stages of collagen and matrix remodeling. In addition, MMPs have proven effects on inflammatory cell migration and infiltration of freshly

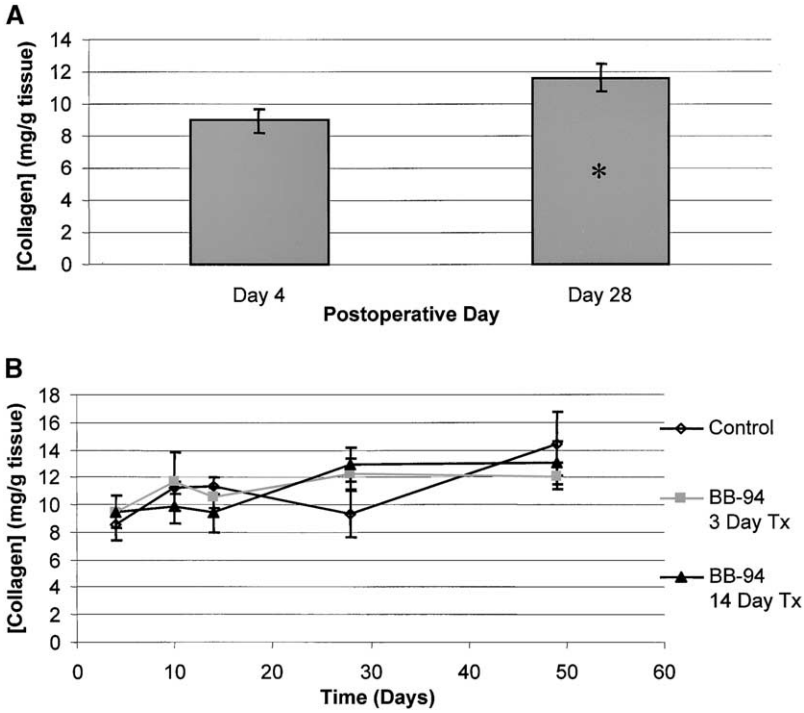


Fig. 2. A, Increase in skin collagen content between 4 and 28 days for all animals combined (9.0 ± 0.8 mg/g tissue at 4 days vs. 11.7 ± 0.8 mg/g tissue at 28 days, $P < 0.05$). **B**, Skin wound collagen concentrations in the three experimental groups over time ($P = 0.94$).

injured tissue and on angiogenesis,[5,21,22] both of which also have implications for healing. The variety of influences on different aspects of healing requires that any study evaluating potentially deleterious effects of metalloproteinase inhibition include functional tests (such as BBP) in addition to morphologic studies.

Perioperative treatment with BB-94 did not have a significant effect on BBP of the small bowel anastomosis in our study. The control BBP measured in our studies compares favorably with the BBP measured in the small and large bowel of operated and nonoperated rats in other published experiments; the range of bursting pressures reported in these other studies was 178 to 244 mm Hg for nonoperated bowel and 180 to 233 mm Hg for anastomosed bowel on postoperative day 7.[23–25] The studies of Syk et al.[11] and Witte et al.[12] examined the effects of MMP inhibition on wound breaking strength in the skin and colon, respectively. Similar to the findings in our experiments, these other studies indicated that MMP inhibition did not impair wound breaking strength. In fact, the studies of Syk et al.[11]and Witte et al.[12] found that the strength of the wound may be increased by MMP inhibition. This slight disparity may be explained by differences in the types of tissues analyzed, measurement techniques, and time points of measurement. We chose to measure the anastomotic strength in the small intestine, whereas Syk et al.[11] examined colonic wounds, and there is compelling evidence that the MMP profile in the small and large bowel may be quite different.[3] Whereas Syk et al.[11]and Witte et al.[12] used tensiometry to measure wound breaking strength, we used increasing intraluminal pressure to interrogate the repair, and this technique may better represent the physiologic demands of a bowel anastomosis. The other studies measured wound breaking strength at single time points in the relatively early stages of wound healing (day 10 in the skin study and days 1, 3, and 7 in the colon study).[11,12] We measured and then analyzed BBP over a range of time periods, which concluded at 7 weeks, the time point classically associated with a maximal gain in wound tensile strength.[26]

The hydroxyproline and trichrome data from our studies provided us with information about the total collagen content of the skin and bowel anastomoses in the experimental animals. The collagen content of the small intestinal anastomoses in these experiments is similar to the collagen content determined in the small and large bowel of operated animals in other studies[27–30]; the range reported for operated bowel at the site of intestinal anastomosis, as determined by similar methods, is 2.01 to 2.36 mg collagen/g tis-

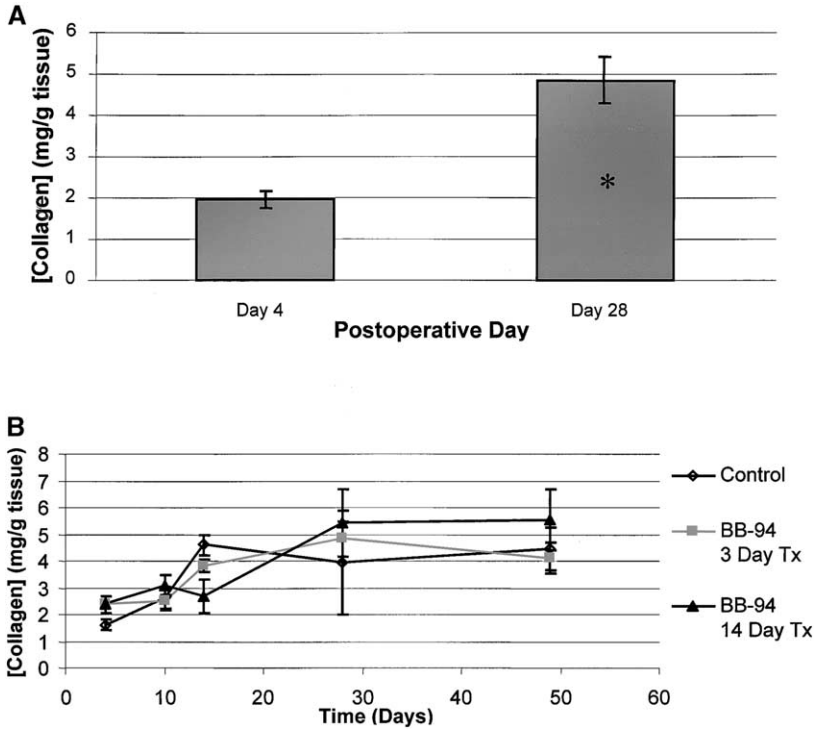


Fig. 3. A, Bowel collagen concentration increase between 4 and 28 days for all animals killed at these time points (2.0 ± 0.2 mg/g tissue at 4 days vs. 4.8 ± 0.6 mg/g tissue at 28 days, $P < 0.001$; *$P < 0.05$). **B**, Bowel collagen content over time in the three experimental groups ($P = 0.63$).

sue.[30] As expected, the skin and bowel collagen concentrations increased from day 4 to day 28 when all animals were considered collectively. This stage in healing represents a relative peak in collagen deposition with a concomitant gain in wound tensile strength.[26] Our study has shown that there was no significant change in the total collagen content of the skin or bowel in rats treated with BB-94, 40 mg/kg, for 3 or 14 days over a 7-week experimental period. Other studies have produced similar results. Witte et al.[12] showed that the collagen content in dorsal skin wounds in rats did not change significantly during treatment with a different MMP inhibitor, GM 6001, and Syk et al.[11] showed that there was no dif-

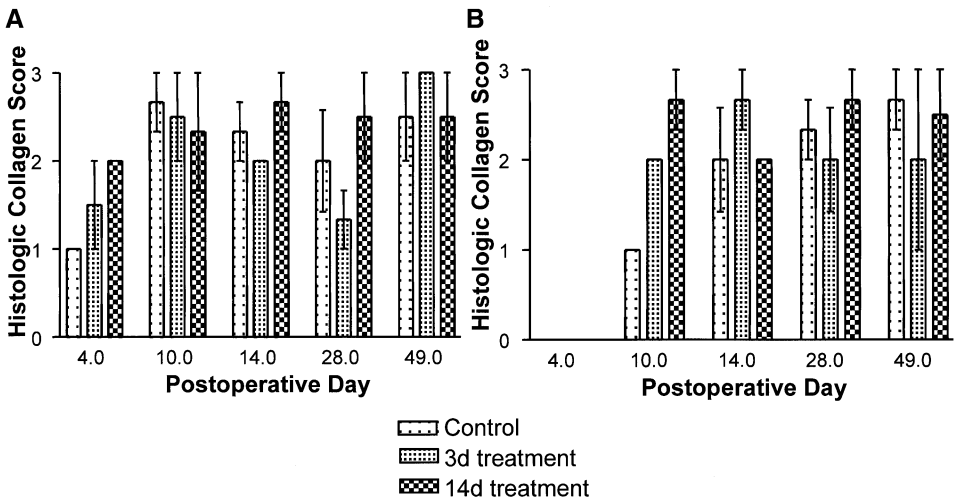


Fig. 4. A, Histologic skin collagen scores in the three experimental groups over time ($P = 0.48$). **B**, Histologic bowel collagen scores in the three experimental groups over time ($P = 0.39$).

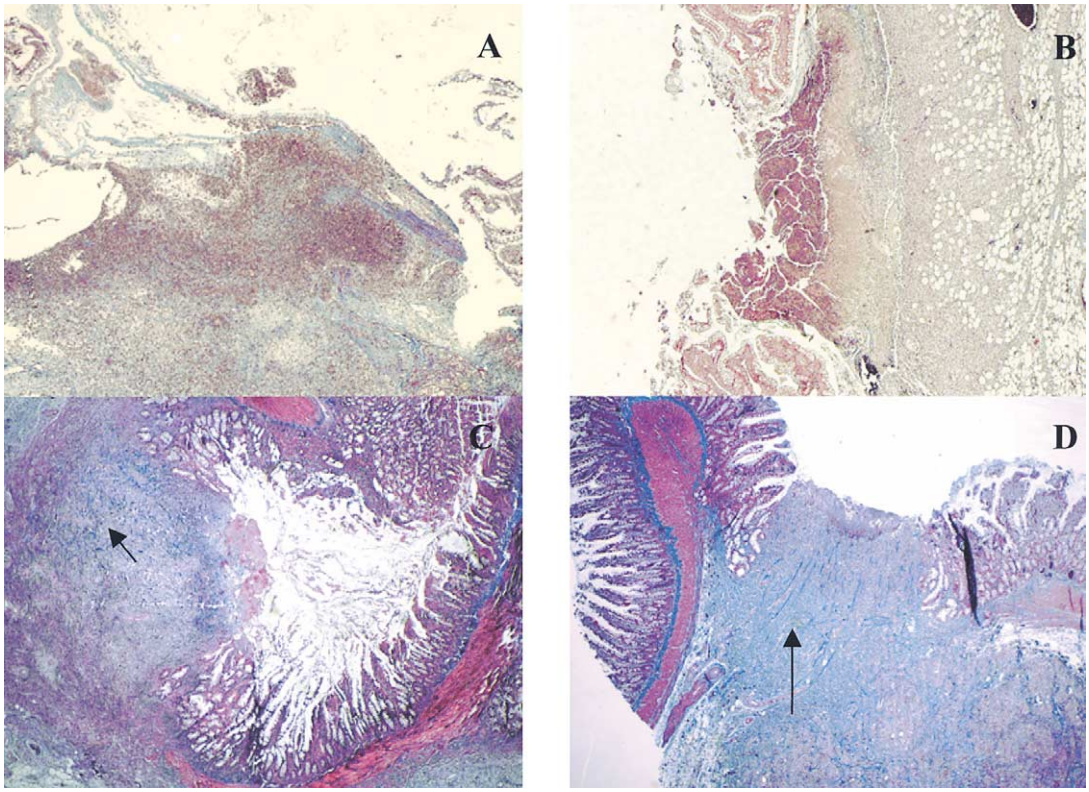


Fig. 5. Collagen trichrome staining at the bowel anastomosis. **A**, Control rat at 4 days. **B**, BB-94–treated rat at 4 days. **C**, Control rat at 49 days. **D**, BB-94–treated rat at 49 days. Note the marked collagen deposition present in the scar at the bowel anastomosis of the control and BB-94–treated rats at 49 days (*arrows*).

ference in the hydroxyproline content of sponges placed in the wounds of rats on postoperative days 3 and 7 compared to control animals. In terms of a mechanistic explanation for these observations, the authors postulate that MMP inhibition leads to an enhancement of collagen maturity and cross-linking and/or some other modulation of the extracellular matrix that strengthens the wound; and Syk et al.[11] pointed out that other studies have shown a beneficial effect for proteinase inhibition without a concomitant increase in collagen content.[21,31] Our data would seem to corroborate these suggestions.

CONCLUSION

We have shown that perioperative MMP inhibition with BB-94 does not impair skin wound or enteric healing in a rat model of laparotomy and small bowel resection. We specifically chose the 40 mg/kg dosage and the 3- and 14-day treatment lengths because they have proven effectiveness in animal models of cancer inhibition.[9,16] Therefore, regardless of differences in specific tissue types and techniques or time points of measurement, this study along with other existing data suggests that the perioperative administration of therapeutic doses of MMP inhibitors is safe with regard to the maintenance of wound strength in oncologic resections.

We thank British Biotech for providing the BB-94. We also thank the Boston Biomedical Research Institute and the Analytical Technology Services, Watertown, Massachusetts, for their assistance with the RP-HPLC and mass spectroscopy.

REFERENCES

1. Soo C, Shaw WW, Zhang X, et al. Differential expression of matrix metalloproteinases and their tissue-derived inhibitors in cutaneous wound repair. Plast Reconstr Surg 2000; 105:638–647.
2. Heath EI, Grochow LB. Clinical potential of matrix metalloprotease inhibitors in cancer therapy. Drugs 2000;59: 1043–1055.
3. Seifert WF, Wobbes T, Hendriks T. Divergent patterns of matrix metalloproteinase activity during wound healing in ileum and colon. Gut 1996;39:114–119.
4. von Lampe B, Barthel B, Coupland SE, et al. Differential expression of matrix metalloproteinases and their tissue in-

hibitors in colon mucosa of patients with inflammatory bowel disease. Gut 2000;47:63–73.
5. Raza SL, Cornelius LA. Matrix metalloproteinases: Pro- and anti-angiogenic activities. J Investig Dermatol Symp Proc 2000;5:47–54.
6. Agren MS. Gelatinase activity during wound healing. Br J Dermatol 1994;131:634–640.
7. Jones L, Ghaneh P, Humphreys M, Neoptolemos JP. The matrix metalloproteinases and their inhibitors in the treatment of pancreatic cancer. Ann N Y Acad Sci 1999;880: 288–307.
8. Deryugina EI, Bourdon MA, Reisfeld RA, Strongin A. Remodeling of collagen matrix by human tumor cells requires activation and cell surface association of matrix metalloproteinase-2. Cancer Res 1998;58:3743–3750.
9. Jimenez RE, Hartwig W, Antoniu BA, et al. Effect of matrix metalloproteinase inhibition on pancreatic cancer invasion and metastasis: An additive strategy for cancer control. Ann Surg 2000;231:644–654.
10. Vaalamo M, Karjalainen-Lindsberg M, Puolakkainen P, et al. Distinct expression profiles of stromelysin-2 (MMP-10), collagenase-3 (MMP-13), macrophage metalloelastase (MMP-12), and tissue inhibitor of metalloproteinases-3 (TIMP-3) in intestinal ulcerations. Am J Pathol 1998;152:1005–1014.
11. Syk I, Agren MS, Adawi D, Jeppsson B. Inhibition of matrix metalloproteinases enhances breaking strength of colonic anastomoses in an experimental model. Br J Surg 2001;88: 228–234.
12. Witte MB, Thornton FJ, Kiyama T, et al. Metalloproteinase inhibitors and wound healing: A novel enhancer of wound strength. Surgery 1998;124:464–470.
13. Rechardt O, Elomaa O, Vaalamo M, et al. Stromelysin-2 is upregulated during normal wound repair and is induced by cytokines. J Invest Dermatol 2000;115:778–787.
14. Colandrea T, Wang L, Wille J, et al. Epidermal expression of collagenase delays wound-healing in transgenic mice. J Invest Dermatol 1998;111:1029–1033.
15. Okada A, Tomasetto C, Lutz Y, et al. Expression of matrix metalloproteinases during rat skin wound healing: Evidence that membrane type-1 matrix metalloproteinase is a stromal activator of pro-gelatinase A. J Cell Biol 1997;137:67–77.
16. Wang X, Fu X, Brown PD, Crimmin MJ, Hoffman RM. Matrix metalloproteinase inhibitor BB-94 (batimastat) inhibits human colon tumor growth and spread in a patient-like orthotopic model in nude mice. Cancer Res 1994;54: 4726–4728.
17. Reddy GK, Enwemeka CS. A simplified method for the analysis of hydroxyproline in biological tissues. Clin Biochem 1996;29:225–229.
18. Edwards CA, O'Brien WD. Modified assay for determination of hydroxyproline in a tissue hydrolyzate. Clin Chim Acta 1980;104:161–167.
19. Wojtowicz-Praga SM, Dickson RB, Hawkins MJ. Matrix metalloproteinase inhibitors. Invest New Drugs 1997;15:61–75.
20. Pilcher BK, Wang M, Qin X, et al. Role of matrix metalloproteinases and their inhibition in cutaneous wound healing and allergic contact hypersensitivity. Ann N Y Acad Sci 1999;878:12–24.
21. Jonsson T, Hogstrom H. Neutrophil-dependent decrease in early wound margin strength. Arch Surg 1991;126:1423–1426.
22. Schwartz JD, Shamamian P, Monea S, et al. Activation of tumor cell matrix metalloproteinase-2 by neutrophil proteinases requires expression of membrane-type 1 matrix metalloproteinase. Surgery 1998;124:232–238.
23. Furst MB, Stromberg BV, Blatchford GJ, Christensen MA, Thorson AG. Colonic anastomoses: Bursting strength after corticosteriod treatment. Dis Colon Rectum 1994;37:12–15.
24. Del Rio JV, Beck DE, Opelka FG. Chronic perioperative steroids and colonic anastomotic healing in rats. J Surg Res 1996;66:138–142.
25. Fukuchi SG, Seeburger JL, Parquet G, Rolandelli RH. Influence of 5-fluorouracil on colonic healing and expression of transforming growth factor-beta 1. J Surg Res 1999;84: 121–126.
26. Lawrence WT. Wound healing biology and its application to wound management. In O'Leary JP, ed. The Physiologic Basis of Surgery. Philadelphia: Williams & Wilkins, 1996, pp 118–140.
27. Jonsson K, Jiborn H, Zederfeldt B. Collagen metabolism in small intestinal anastomosis. Am J Surg 1987;154:288–291.
28. Bowers RL, Eeckhout C, Weisbrodt NW. Actomyosin, collagen, and cell hypertrophy in intestinal muscle after jejunoileal bypass. Am J Physiol 1986;250:70–75.
29. Uden P, Blomquist P, Jiborn H, Zederfeldt B. Impact of long-term relative bowel rest on conditions for colonic surgery. Am J Surg 1988;156:381–385.
30. Kiyama T, Onda M, Tokunaga A, et al. Effect of early postoperative feeding on the healing of colonic anastomoses in the presence of intra-abdominal sepsis in rats. Dis Colon Rectum 2000;43(Suppl 10):54–58.
31. Tani T, Tsutamoto Y, Eguchi Y, et al. Protease inhibitor reduces loss of tensile strength in rat anastomosis with peritonitis. J Surg Res 2000;88:135–141.

Conservative Management of Ingested Foreign Bodies

Steven T. Weiland, M.D., Michael J. Schurr, M.D.

We reviewed the clinical benefits of hospitalization, esophagogastroduodenoscopy, and surgical intervention for ingested foreign bodies in adults. Patients with esophageal foreign bodies were not included in the study group. A 10-year experience is reported. Each patient's physical examination findings at presentation, white blood cell count, length of hospital stay, number and types of foreign bodies ingested, endoscopic interventions, surgical interventions, and complications were reviewed. There were 75 separate hospitalizations, all occurring in 22 male prison inmates. A total of 256 foreign bodies were ingested. Patients incurred 281 hospitalization days (average 3.7 days per admission). One patient had signs of peritonitis. White blood cell count was less than 10 K/μL in 85%. Sixty-four endoscopies were performed with removal of 79 of 163 foreign bodies (48% success rate). Five patients required general anesthesia because of a lack of cooperation. Complications occurred in four of them, one requiring laparotomy. Eight additional laparotomies were performed. One was performed for an acute abdomen on admission and one for the development of an acute abdomen after conservative management. Two were performed to remove metal bezoars. Four additional laparotomies were performed because of surgeon preference. Among the 23 patients admitted and managed conservatively, 77 (97%) of 79 foreign bodies passed spontaneously. One patient required laparotomy. Of the 256 ingested foreign bodies, 79 were removed endoscopically, 71 were removed surgically, and 106 passed spontaneously. The size, shape, and number were not predictive of the ability to transit the gastrointestinal tract. Foreign body ingestion is problematic in prison inmates. With conservative management, most foreign bodies will pass spontaneously. Endoscopy has a high failure rate and is associated with significant complications. Surgical intervention should be reserved for those who have acute conditions in the abdomen or large bezoars. (J GASTROINTEST SURG 2002; 6:496–500.)

KEY WORDS: Endoscopy, prisoners, laparotomy, bezoars

Foreign body ingestion, although problematic in children, is infrequent in adults and when it occurs it is mostly confined to those with psychiatric disorders or to prison inmates.[1,2] Traditional management protocols have focused on hospital admission and extraction of the foreign body.[3] Endoscopic retrieval by means of esophagogastroduodenoscopy (EGD) is often attempted early after admission because of its perceived success rate and safety. However, often the object has already transited the stomach or is otherwise not amenable to endoscopic removal. Patients are commonly referred for surgical extraction after failure of the endoscopic approach. Unfortunately there are limited reports in the literature evaluating the successes, failures, and complications of these modalities. In addition, many objects are reported to spontaneously transit the gastrointestinal tract after failure of endoscopic retrieval.[2,4] As a result, there are no good data to support removal of foreign bodies over conservative management. The goal of this study was to review the clinical benefits of EGD and surgical intervention for ingested foreign bodies in adults and compare their outcomes with those of patients who were managed conservatively.

MATERIAL AND METHODS

The records of all adults requiring admission to the University of Wisconsin Hospital with foreign body ingestion were reviewed over a 10-year period. A comprehensive review of the University of Wis-

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (poster presentation).

From the Department of Surgery (S.T.W., M.J.S.), University of Wisconsin Hospital, Madison, Wisconsin.
Reprint requests: Michael J. Schurr, M.D., Department of Surgery, University of Wisconsin Hospital, 600 Highland Ave., Madison, WI 53792. e-mail: schurr@surgery.wisc.edu

 Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(01)00027-0

consin Hospital medical records database was undertaken to identify foreign body ingestion in patients over 18 years of age. Patients with isolated esophageal foreign bodies were not included. In addition, the records of those managed on an outpatient basis and those with food bolus impaction (e.g., after vertical banded gastroplasty) were not reviewed. Medical records were reviewed for further analysis. Presenting symptoms, physical examination findings, white blood cell counts, and radiographic findings were noted. The length of hospitalization, number and types of foreign bodies ingested, and complications were also reviewed. Outcomes were analyzed for each admission with respect to the management method including EGD, surgical exploration, or conservative treatment.

RESULTS

A review of the medical records yielded 75 separate hospital admissions for foreign body ingestion in 22 patients. All patients were male prisoners who had been previously diagnosed with a psychiatric disorder. The average age was 28 years (range 19 to 47 years). Patients incurred 281 hospitalization days (average 3.7 days per admission) (Fig. 1). Repeat episodes of foreign body ingestion requiring readmission occurred in 14 (64%) of 22 (Table 1). Patients were further analyzed separately for each of the 75 admissions.

Although 37 (49%) of 75 patients complained of abdominal pain on admission, only one presented with signs of peritonitis. The white blood cell count was less than 10 K/μL in 85% of admitted patients (average 8.3 K/μL), and only two patients had a white blood cell count greater than 12 K/μL (13.9 and 25.7 K/μL) (Fig. 2). A total of 256 foreign objects were ingested (average 3.4 objects per admission), with a surprising range of 1 to 50 objects ingested. The number and types of foreign bodies ingested were varied with no particular type, size, or shape of foreign body predominating (Table 2). Abdominal radiographs were obtained from all patients and none showed radiographic evidence of free intraperitoneal air or bowel obstruction.

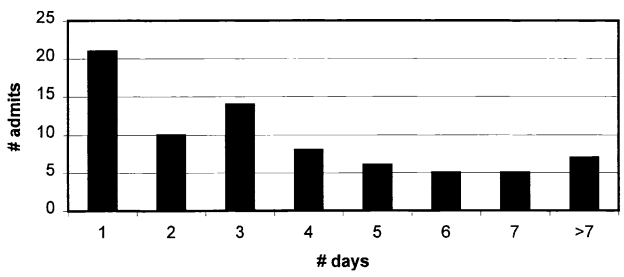


Fig. 1. Length of hospital stay.

Table 1. Summary of 22 patients

Patient	No. of admissions	Total LOS (days)	Total FBs	No. of EGDs	No. of laparotomies
1	1	5	4	0	0
2	1	3	1	0	0
3	1	5	3	0	0
4	1	9	2	0	0
5	1	3	1	1	0
6	1	1	2	1	0
7	1	5	1	0	0
8	1	4	1	1	0
9	2	7	5	3	0
10	2	3	10	2	0
11	2	2	9	2	0
12	2	9	3	0	0
13	2	7	2	1	0
14	3	7	6	3	0
15	3	7	11	3	0
16	3	8	6	2	0
17	4	18	10	11	1
18	4	6	7	4	0
19	5	17	28	1	0
20	6	7	10	7	0
21	13	63	30	11	3
22	16	85	104	11	5

FBs = number of foreign bodies ingested; LOS = length of hospital stay.

Endoscopic Removal

Sixty-four endoscopic examinations were performed in 47 (63%) of 75 patients admitted, with removal of 79 of 163 foreign bodies. This translates into a success rate of 48%. Twenty-four (51%) of 47 patients admitted who were initially treated endoscopically had all foreign objects removed endoscopically. Twenty were successfully treated with a single endoscopic procedure and four required two or three endoscopic procedures. In 23 of 47, the remainder of the foreign bodies had transited past the stomach/duodenum or were considered to be unremovable via the EGD approach. Five patients required general anesthesia because of a lack of cooperation. The use of overtubes during endoscopic foreign body removal is routine at our institution. However, use of overtubes was rarely documented in procedural notes.

Four complications occurred (6%) including three esophageal tears that were treated conservatively with antibiotics, intravenous fluids, and restriction of oral intake; none of these required surgical interven-

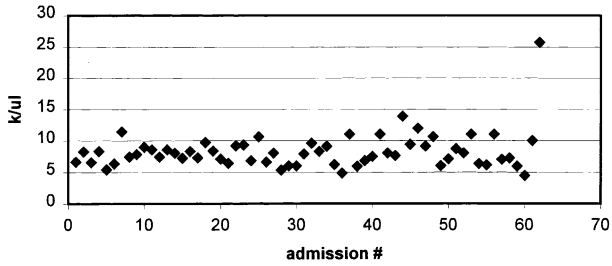


Fig. 2. White blood cell count on admission.

tion. The fourth complication involved retention of an endoscope after a snare was successfully passed around a comb. The comb could not be withdrawn and the snare could not be removed from the comb. The patient required a laparotomy for removal of the retained endoscope-snare foreign body. In addition, one scope was bitten into two pieces and destroyed. Two laparotomies were performed because of the surgeon's preference for removal of a foreign body after failed EGD. The remaining patients were managed conservatively, with passage of the foreign bodies without complications.

Surgical Removal

Five additional laparotomies were performed. One was performed for an acute condition in the abdomen at the time the patient was admitted. This patient had come to the emergency room with obvious signs of peritonitis and a white blood cell count of 25.7 K/μL. Surgical exploration was undertaken emergently to remove the foreign body and repair the intestinal perforation. Two laparotomies were performed to remove metal bezoars (50 pieces of typewriter keys and 13 pieces of antennae) (Fig. 3). Two additional laparotomies were performed according to the surgeon's preference for removal of a single item in an asymptomatic patient. One patient who was treated surgically required readmission for a postoperative ileus that was managed conservatively.

Table 2. Examples of foreign bodies ingested

Broken glass
Light bulb
Toothbrush
Pencil
Radio antenna
Chicken wire
Razor blades
Comb
Nail clipper
Spoon
Typewriter keys
Metal hinges

Conservative Management

Among the 23 patients admitted and managed conservatively, 77 (97%) of 79 foreign bodies were passed spontaneously (Figs. 4 and 5). One patient required laparotomy for the development of an acute abdomen.

Of the 256 ingested foreign bodies, 79 were removed via EGD (31%), 71 were removed surgically (28%), and 106 passed spontaneously (41%). Among patients managed conservatively, 97% of the objects passed spontaneously (Fig. 6). Except for the two patients with gastric bezoars, the size, shape, and number of foreign bodies were not predictive of the ability to transit the intestinal tract (see Figs. 4 and 5).

DISCUSSION

Treatment of foreign body ingestion has traditionally involved hospitalization with close observation until the object is passed. With the development of the endoscope and as techniques have become

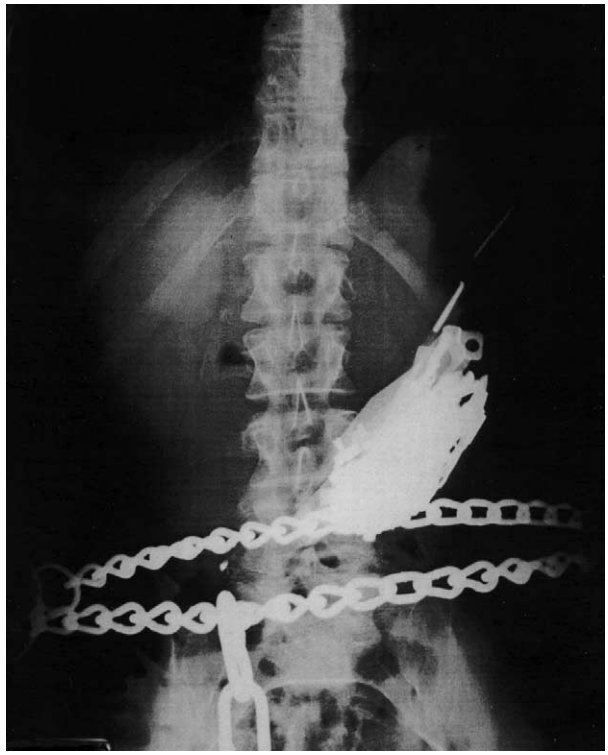


Fig. 3. Metal bezoar from 50 pieces of typewriter keys.

more refined, access to the upper gastrointestinal tract has been facilitated.[3,5,6] Therapy has evolved into increasing use of the endoscope for foreign body retrieval. Currently, some investigators advocate mandatory endoscopy for all patients with a history of foreign body ingestion, despite failure to remove the object in 48%.[4]

This series, although hampered by the retrospective nature of the evaluation, argues against the routine use of endoscopy for the management of foreign body ingestion. Endoscopy has probably developed into this role through its perceived less invasive nature and safety profile. The failure rate of foreign body extraction in this series did not differ from those in previously published series.[4] On the other hand, complete removal of foreign bodies was accomplished in 51% of the patients undergoing endoscopy in this study. The 6% complication rate, however, is cause for concern. If one factors in the need for general anesthesia and the destroyed endoscope, the complication rate becomes 14%, which is significant. Clearly, based on these success and complication rates, the routine use of endoscopy should be discouraged.

The second dilemma with routine attempts at endoscopic removal of foreign bodies arises when the foreign body cannot be removed. Does the failure of one invasive procedure mandate a second more invasive procedure (i.e., laparotomy)? Because of the surgeon's preference, two of our patients underwent elective laparotomy for foreign body removal after failed endoscopy. The remainder were managed conservatively and none developed an acute abdomen. More than half of the foreign bodies in patients initially managed endoscopically passed spontaneously. Clear indications for surgical intervention include signs of peritonitis, bowel obstruction, hemorrhage, or gastric bezoars. Others have also shown that long, sharp objects (see Figs. 4 and 5) can pass spontaneously and do not warrant mandatory removal.[7]

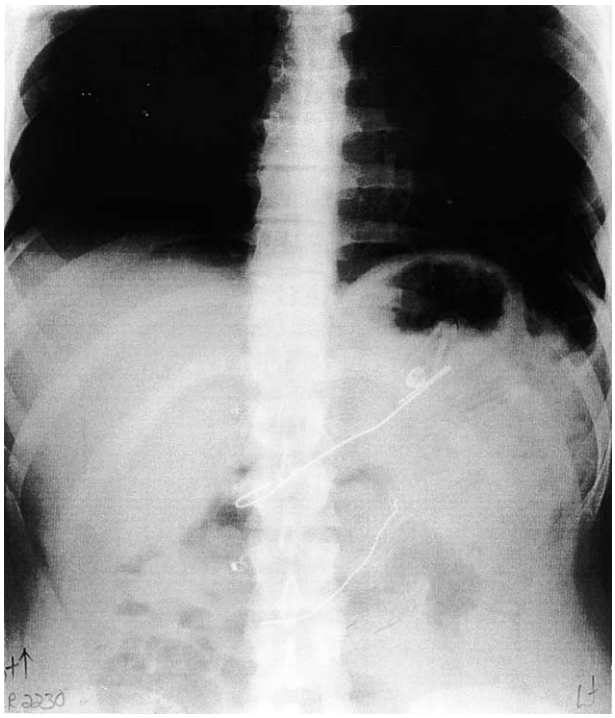


Fig. 4. Long pieces of metal wire that passed spontaneously.

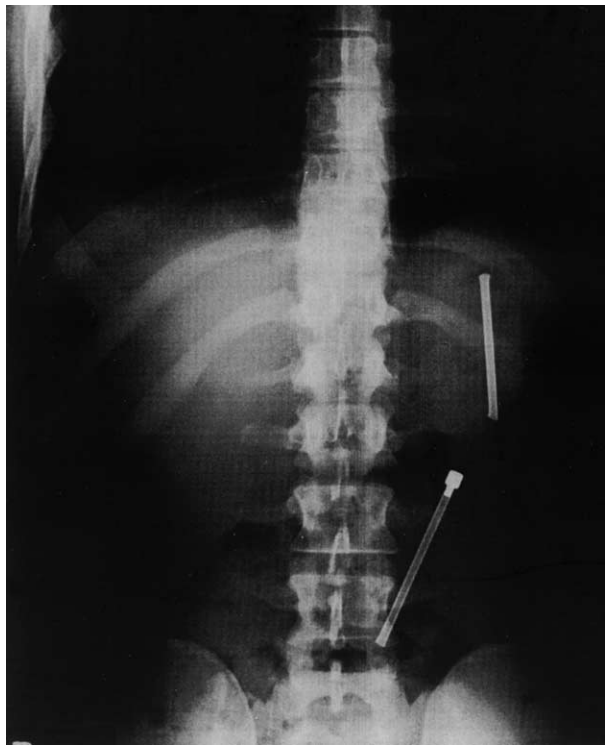


Fig. 5. Long pieces of antennae that passed spontaneously.

Routine conservative management is advocated as the protocol of choice for foreign body ingestion. These data show that the adoption of this approach would allow spontaneous passage of nearly all swallowed objects. Patients who develop a perforation or acute abdomen should be able to be treated surgically without complications if there is no delay in operative intervention, as in the two patients reported here. We would advocate observation of the asymptomatic patient until the object is passed. Whether this takes place in the hospital or at home would depend on the patient's particular living environment. Symptomatic patients who fail to pass foreign bodies out of the stomach after an attempt at conservative management should be considered for endoscopic removal. Objects should be removed through an overtube and with surgical backup in case of complications or inability to remove the foreign body. The timing of the intervention would again depend on

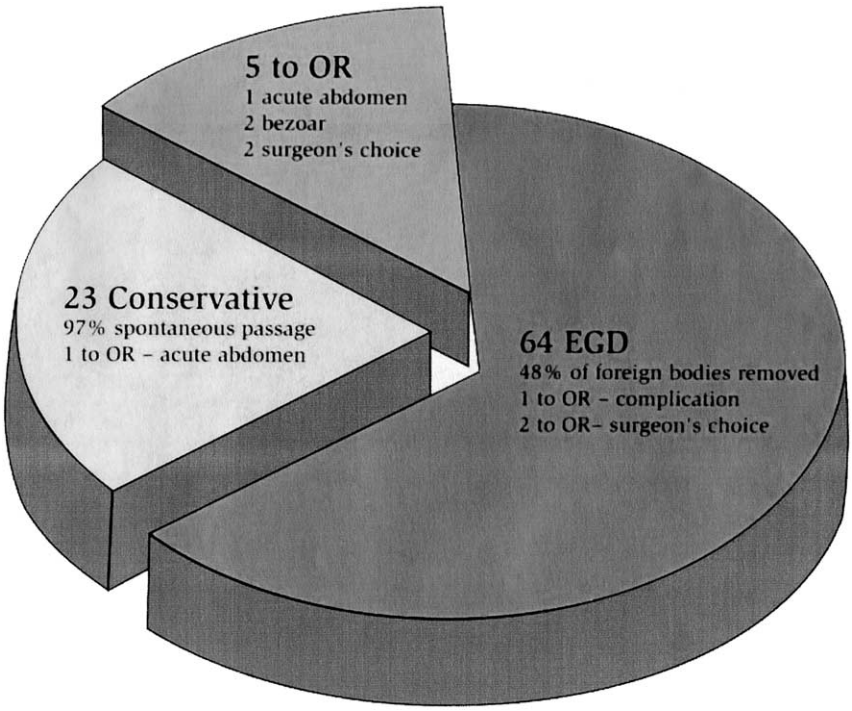


Fig. 6. Summary of foreign body management. OR = laparotomy.

the particular status of the patient and the living environment.

This series did not evaluate patients with esophageal foreign bodies or those who had swallowed caustic substances (e.g., batteries). These entities should be managed with active efforts at foreign body removal because of the risk of airway obstruction and caustic injury to the gastrointestinal tract. In addition, no recommendations for the management of foreign bodies in children can be extrapolated from this series.

There were clearly secondary-gain issues in several of the patients in our unique population. Two of our patients were admitted a total of 29 times and underwent 22 endoscopies and eight laparotomies (see Table 1). One of these patients swallowed a spoon on the day of his latest hospital discharge. We would argue that these patients should be monitored in the prison system to remove all secondary-gain end points in an attempt to discourage this behavior.

CONCLUSION

Foreign body ingestion is problematic in prisoners with psychiatric disorders; however; most of the swallowed objects will pass through the gastrointestinal tract without complications. Indications for surgical exploration of the abdomen include signs of peritonitis, obstruction, hemorrhage, or the unique setting of a foreign body bezoar. EGD is recommended only for isolated cases because of its modest success rate and risk of complications.

REFERENCES

1. O'Sullivan ST, Reardon CM, McGreal GT, Hehir DJ, Kirwan WO, Brady MP. Deliberate ingestion of foreign bodies by institutionalized psychiatric patients and prison inmates. Ir J Med Sci 1996;165:294–296.
2. Losanoff JE, Kjossev KT. Gastrointestinal crosses. A new shade from an old palette. Arch Surg 1996;131:166–169.
3. Barros AL, Caballero A, Rueda JC, Monturiol JM. Foreign body ingestion: Management of 167 cases. World J Surg 1991;15:783–788.
4. Ciriza C, Garcia L, Suarez P, Jimenez C, Romero MJ, Urquiza O, Dajil S. What predictive parameters best indicate the need for emergent gastrointestinal endoscopy after foreign body ingestion? Clin Gastroenterol 2000;31:23–28.
5. McCaffery TD, Lilly JO. The management of foreign affairs of the GI tract. Am J Dig Dis 1975;20:121–126.
6. Mirhej MA, Koch J, Stansell J. A novel approach to ring-type foreign body removal: The "U wire." Gastrointest Endosc 1999;49:243–245.
7. Selivanov V, Sheldon GF, Cello JP, Crass RA. Management of foreign body ingestion. Ann Surg 1983;199:187–191.

Ongoing Deficits in Resident Training for Minimally Invasive Surgery

Adrian Park, M.D., Donald Witzke, Ph.D., Michael Donnelly, Ph.D.

Patient preference has driven the adoption of minimally invasive surgery (MIS) techniques and altered surgical practice. MIS training in surgical residency programs must teach new skill sets with steep learning curves to enable residents to master key procedures. Because no nationally recognized MIS curriculum exists, this study asked experts in MIS which laparoscopic procedures should be taught and how many cases are required for competency. Expert recommendations were compared to the number of cases actually performed by residents (Residency Review Committee [RRC] data). A detailed survey was sent nationwide to all surgical residency programs (academic and private) known to offer training in MIS and/or have a leader in the field. The response rate was approximately 52%. RRC data were obtained from the resident statistics summary report for 1998–1999. Experts identified core procedures for MIS training and consistently voiced the opinion that to become competent, residents need to perform these procedures many more times than the RRC data indicate they currently do. At present, American surgical residency programs do not meet the suggested MIS case range or volume required for competency. Residency programs need to be restructured to incorporate sufficient exposure to core MIS procedures. More expert faculty must be recruited to train residents to meet the increasing demand for laparoscopy. (J GASTROINTEST SURG 2002;6:501–509.)

KEY WORDS: Laparoscopic surgery, minimally invasive surgery, internship and medical residency, surgery, graduate medical education

Cushieri,[1] a visionary and pioneer in the field of minimally invasive surgery (MIS), once commented regarding laparoscopy that rarely in surgical history have we seen so profound a benefit in patient care in so short a period of time. There is no denying that the impact of MIS on patient care since the introduction of laparoscopic cholecystectomy in 1987 has been little short of revolutionary. As the number of techniques performed laparoscopically has exploded over the past decade, patients have benefited from shorter hospital stays, more rapid postoperative recovery, and more rapid return to normal activity than after comparable open procedures. Hospitals too have benefited from MIS by increasing surgical case volumes while reducing the number of inpatient surgical beds. Yet, despite the benefits of MIS, many concerns about patient safety and well-being and challenges related to the lack of generally accepted guidelines have been raised. Because patient demand drove the adoption of early procedures such as laparoscopic cholecystectomy, neither rigorous evaluation nor prospective, randomized comparisons with established "gold standards" of care were carried out. By the early 1990s, general surgeons felt compelled to offer their patients laparoscopic cholecystectomy for fear of otherwise losing referrals for gallstone disease. These social and market forces placed surgeons and their patients in an obviously precarious situation. The neophyte laparoscopist would often have to observe cases or seek training wherever and however possible before undertaking these new procedures.

This new field of surgery did not emanate from academic medical centers; initially the charge to adopt new MIS techniques was led by private practitioners in North America. Academic surgical centers have essentially been playing "catch-up" for the past decade. The ideal context in which MIS training should occur is within a surgical residency. Yet often residency training programs encounter the same difficulties in teaching new techniques (and establishing competency) that are identified by practicing sur-

Presented at the Forty-Second Annual Meeting of The Society for Surgery of the Alimentary Tract, Atlanta, Georgia, May 20–23, 2001 (oral presentation).
From the Departments of Surgery (A.D., M.D.) and Pathology (D.W.), University of Kentucky College of Medicine, Lexington, Kentucky.
Supported in part by an educational grant from Tyco/U.S. Surgical Corporation.
Reprint requests: Adrian E. Park, M.D., Department of Surgery, Center for Minimally Invasive Surgery, University of Kentucky, C343, 800 Rose St., Lexington, KY 40536-0298. e-mail: apark@pop.uky.edu

1091-255X/02/$—see front matter
PII: S1091-255X(02)00021-5

geons. Despite the increasing move toward MIS procedures in surgical practice, no consistent model for training the practicing surgeon in new MIS techniques or for assessing competency has yet been developed.[2]

The purpose of this study was to glean a consensus from leading experts in MIS regarding which procedures should be incorporated into residency training and to determine the number of cases that would be necessary to achieve competency in them.

METHODS

A questionnaire related to minimally invasive surgery procedures (MISQ) was distributed to two samples. The first sample was drawn from a list of 48 surgeons at institutions known to have a program in MIS and/or known as leaders in the field. Twenty-five surgeons who returned their forms (52% return rate) were included in the study. The second group was a convenience sample of 14 individuals who attended MIS workshops at the University of Kentucky or who were volunteer residents and faculty. The questionnaire used to collect data for the current study was divided into three sections. The first section required respondents to indicate the number of minimally invasive laparoscopic cholecystectomies and laparoscopic inguinal herniorrhaphies a surgical resident must perform as the primary surgeon to be fully competent in both. In the second section, respondents were asked to indicate how many cases a resident must perform as the primary surgeon to be competent in other common procedures, given prior competency in laparoscopic cholecystectomy and laparoscopic inguinal herniorrhaphy. The third section requested that surgeons indicate whether all general surgeons should be able to perform the MIS procedure ("Should it be a core MIS procedure?").

Data regarding the average number of MIS procedures actually performed by residents in 1998–1999 were also obtained from the Accreditation Council for Graduate Medical Education (ACGME) *Surgery Resident Statistics Summary* by the Residency Review Committee (RRC) for Surgery.[3] The statistics were based on reports for 936 residents in 240 programs nationally as of November 23, 1999. An updated version (989 residents in 252 programs as of April 3, 2001) of the data matrix was downloaded from the ACGME web site.[4] Statistical analyses were computed for both sets of data. A third set of data obtained from a report published by the Millenium Research Group provided estimates of the number of MIS procedures done nationally by all surgeons for 1998–1999.[5] Data for only five procedures (laparoscopic appendectomy, laparoscopic antireflux procedures, laparoscopic cholecystectomy, laparoscopic colon/intestinal resection, and laparoscopic inguinal herniorrhaphies) were available to be used in our comparisons.

Responses from experts were compared to those from nonexpert surgeons to determine whether differences would be so large as to preclude using all replies from all respondents to characterize MIS procedures. Data regarding the number of MIS procedures a resident needed to perform to be considered competent were compared for the two groups using *t* tests. Similarly, the number of respondents in each group who indicated whether a procedure should be core or not was compared using χ^2 analysis. Because two respondents made a "Yes" and a "No" response to a MIS procedure, the ratings were designated as "Yes" only for comparisons involving frequency.

Data from the RRC reported for both periods were compared statistically to MISQ data using *t* tests. Bonferroni corrections were made for all statistical comparisons, using 50 as the number of variables compared for the entire study. This correction adjusts the alpha level required for a *P* value to be significant to reduce the probability that the finding was a chance result because of the number of significance tests that were calculated. Where statistical significance was obtained after the Bonferroni correction, Cohen's effect size was calculated for each comparison to determine the "importance" of the significant finding.

A one-way analysis of variance was used to determine if there were significant differences in the mean number of times the various procedures needed to be performed for competency. Although a within-groups design would normally have been appropriate to test the differences among the means, a between-groups design was used instead. The impact of this design change was to make our testing of the null hypothesis more conservative; that is, we increased the probability that we would accept the null hypothesis when it was, in fact, false. The Games-Howell post hoc test was used to identify the exact pattern of differences among the means. This method was chosen because it can be used with unequal numbers of subjects and heterogeneous variances.[6] Both of these conditions were present in our data.

Single-group *t* tests for proportions were used to determine if procedures were judged "core" by the group as a whole.[7] The proportion of MIS surgeons designating a procedure as core was compared to a hypothetical population mean of 0.5, the expected mean if no consensus emerged. If the proportion was significantly higher than 0.5, we considered it to be a

core procedure. If the proportion was significantly lower than 0.5, it was not considered core.

RESULTS

Experts vs. Nonexperts on the MIS Questionnaire

Comparisons of both groups' estimates of the number of MIS procedures required for competency yielded no significant differences after the Bonferroni correction. Although not significant after the correction, means (and standard errors of the mean) for the two groups for laparoscopic inguinal herniorrhaphies were 42.8 ± 4.1 and 25.7 ± 3.5 (t = 2.79, df = 37), respectively. Although estimates of the number of procedures required to be competent in laparoscopic cholecystectomy (n = 200) and laparoscopic inguinal herniorrhaphies (n = 100) made by one expert might be considered outliers, we made no adjustment. The means, standard errors of the mean, and numbers responding for laparoscopic cholecystectomy and laparoscopic inguinal herniorrhaphies are displayed in Table 1. Data for the number of procedures required for competency for the remainder of the procedures are also presented in Table 1. Note that sample sizes varied because of missing data. None of the χ^2 analyses resulted in significant differences between the two groups regarding the percentage of respondents indicating that the procedure should be considered as core in training residents.

Because there were no significant differences in the estimates of experts and nonexperts of the number of times a procedure needed to be performed for competency, the two sets of estimates were combined in the following analyses. A between-groups analysis of variance was performed to determine if there were overall significant differences in the frequency with which the various procedures needed to be performed to achieve competency. Laparoscopic cholecystectomy and laparoscopic inguinal herniorrhaphies were not included in this analysis, because they were assumed to be the first procedures to be learned and thus required a steeper learning curve. The analysis of variance was significant (F = 12.90, df = 10, 394; $P < 0.0001$). The Games-Howell post hoc test was used to determine the exact pattern of differences. The number of times the various procedures needed to be performed could be divided into three levels. The means needed for competency in the relatively high-frequency laparoscopic procedures are bariatric (33.2), colon (27.6), and antireflex (24.0). Biliary (21.3), gastric (20.0), and adrenal (17.2) are at the second level and need to be performed between 17 and 21 times for competency. The lowest group includes spleen (14.8), anterior/incisional hernia (12.9), gastrostomy/jejunostomy (10.8), diagnostic procedures (10.8), and appendectomy (10.6), which need to be performed between 11 and 15 times for competency.

Experts vs. Residency Review Committee Procedure Report

Comparison of experts' estimates of the number of procedures a resident must perform to be competent with the actual number performed by residents at programs reviewed by the RRC reached significance for all 10 of the procedures listed for 1998–1999.[3] Nine of the 10 procedures reached signifi-

Table 1. Number of procedures as primary surgeon required for competency and number given competency at laparoscopic cholecystectomy and inguinal herniorrhaphy (mean ± SEM)

Procedures	Survey respondents					
	Experts	N	Nonexperts	N	All	N
Laparoscopic inguinal herniorrhaphy (LIH)	42.8 ± 4.1	25	25.7 ± 3.5	14	36.7 ± 1.6	39
Laparoscopic cholecystectomy (LC)	40.4 ± 7.2	25	30.2 ± 4.7	14	36.7 ± 4.9	39
Given competency at LIH and LC						
Laparoscopic bariatric surgery	35.3 ± 6.6	19	29.5 ± 4.5	11	33.2 ± 4.4	30
Laparoscopic colon/intestinal resection	29.4 ± 3.9	25	24.2 ± 4.1	13	27.6 ± 2.9	38
Laparoscopic antireflux procedures	26.2 ± 2.4	25	19.6 ± 4.0	13	23.9 ± 2.1	38
Laparoscopic biliary surgery	22.5 ± 3.4	24	19.0 ± 2.4	12	21.3 ± 2.4	36
Laparoscopic gastric surgery	19.4 ± 2.3	24	21.3 ± 3.5	12	20.0 ± 1.9	36
Laparoscopic adrenalectomy	16.4 ± 1.5	25	18.8 ± 3.4	13	17.2 ± 1.5	38
Laparoscopic splenectomy	15.0 ± 1.5	25	14.4 ± 2.0	13	14.8 ± 1.2	38
Laparoscopic anterior/incisional hernia	13.0 ± 1.4	25	12.7 ± 2.5	12	12.9 ± 1.2	37
Diagnostic laparoscopy	11.4 ± 2.2	25	9.5 ± 2.2	13	10.0 ± 1.6	38
Laparoscopic appendectomy	11.2 ± 0.9	25	9.4 ± 1.6	13	10.6 ± 0.8	38
Laparoscopic gastrostomy and jejunostomy	10.1 ± 1.0	25	12.0 ± 2.5	13	10.8 ± 1.6	38

Table 2. Data for the number of procedures required for competency

Procedures	Data sources			
	Experts	N	RRC	N
Laparoscopic inguinal herniorrhaphy	42.8 ± 4.1	25	7.6 ± 0.3	989
Laparoscopic cholecystectomy	40.4 ± 7.2	25	84.0 ± 1.1	989
Laparoscopic bariatric surgery	35.3 ± 6.6	19	0	989
Laparoscopic colon/intestinal resection	29.4 ± 3.9	25	1.9 ± 0.1	989
Laparoscopic antireflux procedures	26.2 ± 2.4	25	8.5 ± 0.3	989
Laparoscopic biliary surgery	22.5 ± 3.4	24	0.9 ± 0.1	989
Laparoscopic gastric surgery	19.4 ± 2.3	24	0.1 ± 0.0	989
Laparoscopic adrenalectomy	16.4 ± 1.5	25	0	989
Laparoscopic splenectomy	15.0 ± 1.5	25	1.0 ± 0.0	989
Laparoscopic anterior/incisional hernia	13.0 ± 1.4	25	0	989
Diagnostic laparoscopy	11.4 ± 2.2	25	4.7 ± 0.1	989
Laparoscopic appendectomy	11.2 ± 0.9	25	5.4 ± 0.2	989
Laparoscopic gastrostomy and jejunostomy	10.1 ± 1.0	25	1.4 ± 0.1	989

Experts' estimates of the number of procedures as primary surgeon required for competency for laparoscopic cholecystectomy and laparoscopic inguinal herniorrhaphy and number of procedures as primary surgeon required for competency in laparoscopic cholecystectomy and laparoscopic inguinal herniorrhaphy versus number of procedures reported for all residency programs in the April 2001 RRC report.

cance for comparisons of the updated data reported on April 4, 2001 for programs reporting in 1998–1999.[4] All differences that were significant (after the Bonferroni correction) yielded large effect sizes as labeled by Cohen. The single laparoscopic procedure failing to reach significance in the 2001 RRC report was for antireflux procedures.[4] Note that in all comparisons but one (laparoscopic cholecystectomy), MIS experts indicated that residents needed to complete significantly more laparoscopic procedures than the RRC-reported residents were actually doing for both reporting periods.[3,4] In contrast, residents were performing almost twice as many laparoscopic cholecystectomies as experts thought were required. The differences between what the MIS experts estimated as the minimum number required and what residency programs reported to the RRC in 1998–1999 are displayed in Table 2. Five procedures, (laparoscopic bariatric surgery, laparoscopic gastric surgery, laparoscopic adrenalectomy, laparoscopic splenectomy, and laparoscopic anterior/incisional herniorrhaphy) included in the MISQ were listed as having an average of one or fewer cases across residency programs in the 2001 RRC report.[4]

Experts vs. Procedures MIS Performed in 1998–1999

Searching for published data regarding the total number of MIS procedures performed revealed only one source. We have summarized the Millennium Research Group estimates[5] provided for laparoscopic inguinal herniorrhaphy, laparoscopic cholecystectomy, laparoscopic colon/intestinal resection, laparoscopic appendectomy, and laparoscopic antireflux procedures in Fig. 1. Note that although the data sets are different in scale, comparing proportions within data sets allows one to see the difference in relative magnitude among the procedures.

In the MISQ, the MIS surgeons were also asked to judge whether each of 11 procedures was core. The proportion judging a procedure as core was compared to a proportion of 0.5, the expected proportion if no group consensus emerged. Seven of the procedures (diagnostic laparoscopy, laparoscopic antireflux procedures, laparoscopic appendectomy, laparoscopic gastrostomy and jejunostomy, laparoscopic anterior/incisional herniorrhaphy, laparoscopic biliary surgery, and laparoscopic colon/intestinal resection) were judged to be core (all $P < 0.001$), laparoscopic bariatric surgery and laparoscopic adrenalectomy were considered not core (both $P < 0.01$), and there was uncertainty about whether or not laparoscopic splenectomy and laparoscopic gastric surgery were core.

DISCUSSION

Competency, as Trunkey and Botney[8] have pointed out, is a concept that defies easy definition. For surgeons, in addition to varying degrees of medical knowledge, judgment, and inductive reasoning, competency requires visiospatial facility, manual dexterity and, of course, experience. In an earlier study,[9] we tested the hypothesis that judgment was a critical feature of competent surgical performance. Our data demonstrated the need for clinical judgment in the perfor-

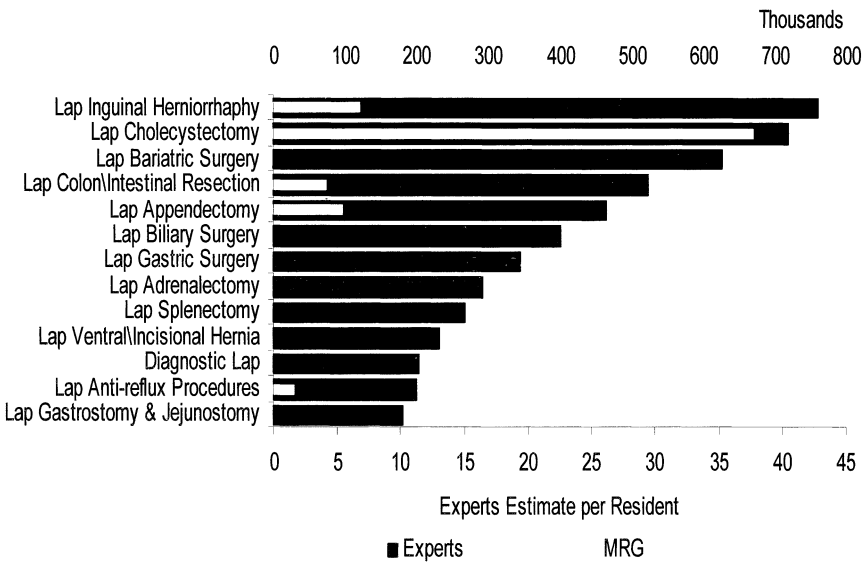


Fig. 1. Experts (N = 25) and Millennium Research Group (*MRG*) estimates of minimally invasive surgical procedures performed in 1998–1999.

mance of laparoscopic skills. This fact may attenuate the learning curve of a skill to a degree that could not be predicted from the purely technical aspects of that skill.[9]

Surgical experience is acquired by both direct and indirect means. Of the many factors that contribute to a surgeon's experience, one that is easily quantifiable is the number of times a surgeon has performed a particular procedure. Although case volumes do not tell the whole story of a surgeon's competency, the American Board of Surgery uses this measure to set minimum normative standards for defined categories of surgical procedures. These numbers are in turn applied by the RRC in assessing surgical residency training programs for accreditation. Although the ACGME has recently (1999) endorsed six additional areas of general competency deemed applicable to all physicians, there are currently no universally accepted measures for any of these suggested areas of competency.[3] At present, in the surgical specialties the number of cases performed by a resident is the most important and widely accepted measure of competency.

A number of forces are currently pressuring the health care training systems in the United States to shift their objectives from being process oriented to being outcome oriented. One force compelling this change is the report from the Institute of Medicine[10] on medical errors. In looking at a comprehensive approach to reducing medical errors and improving health care, the Institute states that no one change will alter the pattern of nonresponsiveness of the health care system. One of the key changes the Institute does address is "identifying and learning from errors."[10] They recommend that "health professional licensing bodies should implement periodic reexaminations and relicensing of doctors . . . based on both competence and knowledge of safety practices . . ."[10] The shift to training outcomes as a basis for accreditation initiated by the ACGME in conjunction with the American Board of Medical Specialties will have direct consequences for MIS training programs. The shifts from process to outcome also carries with it a series of methods (an assessment "toolbox") for evaluating the effectiveness of training in terms of resident performance. Typically, evaluation is characterized by one or more methods that will reliably and validly yield scores that can be used to judge competency and provide sufficient objective information to correct flawed performance by a resident.

IMPLICATIONS

Our data show little disagreement among the experts as to the number of cases required for competency (for the various procedures), or even a significant difference between experts and nonexperts. On the other hand, the disparity between the number of cases recommended by experts and the actual national average number of those cases performed through the course of a surgical residency[3,4] is remarkable. Furthermore, the national average number of cases per graduating surgeon is one or less[3,4] for four procedures (laparoscopic gastrostomy and jejunostomy, laparoscopic anterior/inci-

sional hernia, laparoscopic biliary surgery, and laparoscopic splenectomy) that MIS experts considered core surgical residency procedures.

It might be argued that this "crisis in training" is a fabrication by those proponents of MIS who overstate the real world impact/growth of these procedures. It is very difficult to access current data on the number of laparoscopic procedures being performed nationally or to establish what proportion of specific procedures (e.g., colon resection) is performed laparoscopically. The Millennium Research Group[5] has recently documented current case volumes and projected growth (positive or negative) of various MIS procedures. The Millennium Research Group study notes that cases of MIS are on the rise and that most procedures, with the exception of laparoscopic inguinal herniorrhaphy, are projected to grow over the next few years at annual rates varying from 3% to 15%.[5] These procedures are clearly here to stay, as evidenced by growing patient demand for them. Therefore concerns for the limitations of surgical residency training should not be confined solely to those proponents of MIS.

To bridge the considerable chasm between the recommended number of MIS cases and the actual MIS training experience of graduating surgeons, certain challenges must be met. Given the call from multiple sources for competent performance of physicians, we must ask how to maintain current training quality while implementing broader training in MIS. In response, bold steps need to be considered and taken. There are several reasons why MIS experience for surgical residents is lacking, particularly at academic medical centers. First, most academic surgical departments across the country are still lagging behind the (surgical) private practice community in terms of embracing MIS procedures. Many academic medical centers have only recently recognized the need to establish centers or services in MIS. Second, because attempts at reassigning existing faculty in the hope of developing "homegrown" programs in MIS have often met with failure, emphasis needs to be placed on the recruitment of fellowship-trained laparoscopic surgeons to help build MIS into surgical training programs. Third, support for the training of existing faculty in these new techniques is needed.

Another challenge facing surgical training programs is integrating MIS into an already crowded postgraduate curriculum. None of the demands for training in open surgery have lessened. Because conversion to open surgery is the solution of choice when unanticipated intraoperative complications arise, MIS training must be built on the traditional grounding in open surgery. Even so, more time needs to be reserved for early and repeated resident exposure to MIS. This may occur on specialized services, or by integrating (with appropriate faculty recruitment and retraining) MIS procedures into established services.

Added to the difficulty of integrating MIS into resident rotations is the growing cost of training surgeons in the operating room. This expense (now estimated at $48,000.00 per graduating resident)[11] indicates that more training needs to move out of the operating room. Fortunately, the acquisition of essential laparoscopic skills can be facilitated by the use of mechanical and computer-based simulators. Scott et al.[12] have demonstrated that the skills acquired by practicing simple tasks in a laparoscopic trainer can be transferred to the operating room. The experts in this study indicated that a rather large number of procedure-specific experiences are required for competency; could this number be reduced if the skills needed for MIS were sufficiently honed before the resident entered the operating room? If the resident spends enough time in practice to evidence an adequate level of competency, moving from simulation to an actual patient becomes an efficient and manageable step. Consequently, residency programs will need to allocate time for inanimate or skills laboratory training, as well as provide accessible equipment within reasonable proximity to the operating room and with extended hours of operation.

CONCLUSION

Clearly, the problem of training programs that do not meet training needs is not confined to the field of MIS. Although a full analysis of such problems facing all surgical specialties is beyond the scope of this report, it is hoped that some of the issues raised and suggestions made to address the shortcomings of training in MIS may also find application in other areas of surgical training. We felt the need to focus attention on the inadequacy of resident training in MIS because much is at stake for the general surgeon. The field of general/abdominal surgery has evolved considerably over the past few years. Patients are increasingly "voting with their feet" for minimally invasive procedures in the treatment of gastrointestinal and other abdominal disorders. This is the future of general surgery. If these procedures continue to fall into the domain of the (relatively few) fellowship-trained minimally invasive surgeons, then general surgery faces the very real risk of "devolving" to gallbladder, hernia, and breast surgery along with some miscellaneous procedures.

Current MIS training, as reported by American

surgical residency programs, does not include all of the MIS procedures that experts in the field consider core to surgical residency training. For most core procedures, residents do not carry out the number of those procedures experts say are needed to achieve competency, according to American surgical residency program reports. The solution to this dilemma will be multifaceted, but must include strategies for the recruitment of fellowship-trained laparoscopic faculty to academic medical centers and American surgical residency programs, as well as early and repeated exposure to MIS clinical rotations. More emphasis must be placed on the acquisition of surgical skills outside the operating room by the use of inanimate simulators. Finally, surgical educators must be prepared to explore and harness new technology (virtual reality, imaging, haptics, the Internet, etc.) for the future training of our surgical colleagues.

REFERENCES

1. Cushieri A. Whither minimal access surgery: Tribulations and expectations. Am J Surg 1995;69:9–19.
2. Rogers DA, Elstein AS, Bordage G. Improving continuing medical education for surgical techniques: Applying the lessons learned in the first decade of minimal access surgery. Ann Surg 2001;233:159–166.
3. Residency Review Committee for Surgery. Residency statistical analysis report. In Program Data: Defined Categories. Accreditation Council for Graduate Medical Education, 1999.
4. Residency Review Committee for Surgery. Surgery Resident Statistics Summary (Accreditation Council for Graduate Medical Education web site). Available at http://www.acgme.org/RRC/reports/GSRptC19992000.pdf.
5. Millennium Research Group. US laparoscopy 2000 (Report USLAP00). Toronto, Ontario: Millennium Research Group, 2000.
6. Kirk RE. Experimental Design: Procedures for Behavioral Sciences, 3rd ed. Belmont, California: Wadsworth Publishing, 1994.
7. Hayes WL. Statistics, 5th ed. Ft. Worth, Texas: Harcourt Brace, 1994.
8. Trunkey DD, Botney R. Assessing competency: A tale of two professions. J Am Coll Surg 2001;192:385–395.
9. Donnelly MB, Witzke DB, Mastrangelo M, Park A. The educational implications of the importance of clinical judgment in performing laparoscopic skills. Poster presented at SAGES 2000.
10. Kohn LT, Carigan JM, eds. To err is human: Building a safer health system. Washington, D.C.: N.A. Press, 1999.
11. Bridges M, Diamond DL. The financial impact of teaching surgical residents in the operating room. Am J Surg 1999;177:28–32.
12. Scott DJ, Bergen PC, Rege RV, Laycock R, Tesfay ST, Valentine RJ, Euhus DM, Jeyarajah DR, Thompson WM, Jones DB. Laparoscopic training on bench models: Better and more cost effective than operating room experience? J Am Coll Surg 2000;191:272–283.

Discussion

Dr. G. Branum (Harrisonburg, VA): I was wondering whether in the course of your study you were able to gather any statistics from minimally invasive fellowship programs and whether all of the fellowship programs are meeting all of these levels in areas of competency?

Dr. A. Park: That is an interesting question and probably warrants a follow-up study. Although many of the respondents, in fact, ran fellowship training programs, our focus was solely on the resident training experience.

Dr. L.W. Way (San Francisco, CA): I have two questions. First, if you exclude such things as bariatric surgery and adrenalectomy (and maybe Heller myotomy might be on that list) from the course of study for general surgical training, then we really do not have any formal training for those procedures. I think that, in general, the profession accepts that training in bariatric surgery is part of a general surgical education today. So that creates a special problem of how you are going to deal with the excluded group.

My second question concerns the problem of the mismatch between the ultimate intended careers of the learners and the pool of available teaching material. We continue, in general surgery residency programs, to train people, at the time they are chief residents, to do things that they never intend to do in the future, and we find that in this particular instance there is just a shortage of training material. If we could resolve this issue of mismatch, it seems to me that this might be another way to approach this problem.

Dr. Park: I will respond to your second question first. One of the points that we make in our article is that lest you think that this is a shortage or crisis fabricated by proponents of minimally invasive surgery, we very diligently sought data that would help us determine whether the training material—that is, the clinical base or volume, if you will—was growing or not, and whether this is a real or simply a contrived problem.

Again, this is addressed in more detail in our report, but one commissioned study that was published last year looked at the growth rates of these various procedures and, with the exception of one interestingly, laparoscopic inguinal herniorrhaphy—which is currently in the negative growth phase and is projected to remain so for the next year or two, every other procedure is projected to grow from 3% to 15%

per annum, including all of the procedures that we included in our study, for the foreseeable future. So I think we are focusing on areas where there is patient demand and clinical need.

Dr. M.T. Dayton (Salt Lake City, UT): I do not think the phenomenon you have described here is limited to minimally invasive surgery. For example, if you take into consideration "open" surgical cases, such as thoracic procedures, ENT procedures, and maybe some of the more complicated biliary and hepatic cases, many of the residents in our programs finish their training without performing an adequate number of operations to really be described as competent. So this is really not a phenomenon that is limited to the domain of minimally invasive surgery.

Dr. Park: This could apply to other areas as well. Obviously, again, we focused specifically on minimally invasive surgery. This is a specified field with a much shorter history that has brought our attention to the problem. But certainly this could apply on a broader basis.

Dr. C. Pellegrini (Seattle, WA): I believe the answer is simple. If you want to be practical today in the United States, you will have to either close a number of residency programs and just use a few that will have a sufficiently high volume to accomplish the things that you plan or it will be necessary to simply extend the training of a surgeon, as is done in Europe, for 10 years or 20 years, neither of which society in this country would be willing to do. So if you want to be practical, you would have to stay with the numbers that we have right now. That is my personal opinion. I am not speaking for the Accreditation Council for Graduate Medical Education.

But my question is, were these surgeons instructed as to what might be an outcome to look for when they decided that the number of cases should be 30 and not 15 or 20? Was it when time plateaus or when complications plateau? Why did they decide that this was the number of operations that a person needed to be trained? Second, did you ask them what the number of operations per year might be that a surgeon might have to perform throughout his or her career in order to remain competent? As to the American Board of Medical Specialties part of the outcomes projects that you alluded to, this board requires that for recertification purposes or maintenance of certification, a person will have to meet these criteria. It is a remarkable comparison if, as you stated today, 30 colectomies would be required compared to the number that is currently required for recertification, which is eight.

Dr. Park: No, we did not ask about the ongoing process. That is a good point. The respondents to our survey were not asked to elaborate upon the reasons they determined a particular "number of cases" conferred competency for a specific procedure. The number may have reflected a time or complications plateau but more likely was a global assessment or rating.

Dr. Pellegrini: How about maintenance?

Dr. Park: No, we did not look at that either. Again, we focused, just as in a snapshot, on the subject of residency training.

Dr. L. Rikkers (Madison, WI): My comment is along the line of Dr. Dayton's in that with index cases that are used in surgery, including laparoscopic surgery, there are not enough cases nationwide to train everyone who is being trained. So that is a problem we need to address.

There is another element to this, however, and that is the transference of skills. For example, if a surgeon has the opportunity during training to perform 20 major liver resections but happens not to encounter any proximal bile duct cancers, he or she is going to be able to learn how to handle a proximal bile duct cancer much better if he or she has a broad experience in liver resection.

I would ask you, how about this transference as it relates to the field of laparoscopy? For example, if you are in a program that is very rich because there is a large hematology unit on the medical side in doing elective laparoscopic splenectomies, do trainees who learn how to perform that operation well need as many cases to learn how to perform a laparoscopic adrenalectomy after they become proficient in laparoscopic splenectomies? I think that is the additional factor of how skills are transferred from one procedure to another or how persons are able to learn another procedure quite easily if they have performed a procedure that is fairly similar a number of other times.

Dr. Park: The purpose of this study was to present a "snapshot view" and to basically raise issues that would instigate discussion. The issue of transfer of training is a very important one, and we have addressed in the manuscript not only transferring skills from laparoscopic splenectomy to adrenalectomy, but we believe increasingly that skills can be acquired (transferred from) outside the operating room. There are several centers across the country and efforts at uniting focus across the country right now on the development of some of these psychomotor skills, specifically in this area, such that the number of procedures that need to be performed in the operating room can drop precipitously from 30 colectomies down to a much more manageable number. So we agree very definitely that there is opportunity for transfer, not just from other techniques but from nonoperative training experiences.

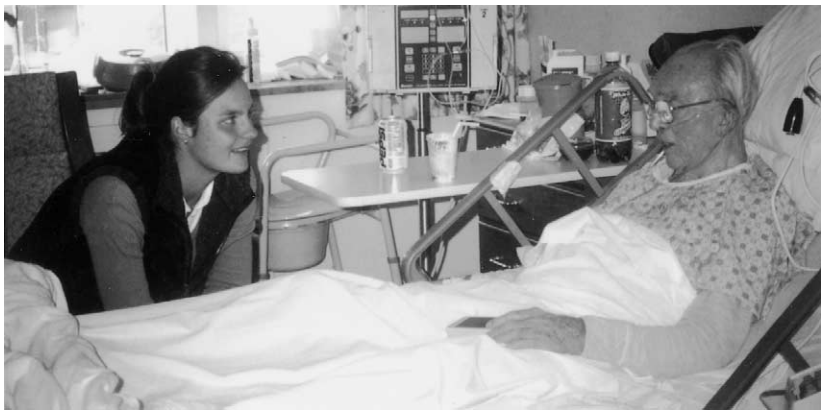
Dr. B.D. Schirmer (Charlottesville, VA): I have a comment and then another question, both of which echo points made by Dr. Rikkers. The comment I want to make is that I do not think we should be such alarmists about this situation; if you look at the numbers reported by the RRC 5 years ago, the mean number of Nissen procedures being done was one and the hernia repairs was down around one or two. So within 5 years we have increased those numbers significantly. Thus, I think it is very clear that the problem is that we as faculty at institutions where residents are being taught are just not seeing as many cases, and if we were seeing more of these cases, we would have the residents performing more operations. So I think we are making some improvements, but clearly it is a very slow process.

My second question again echoes the question of whether you asked the experts if they had a number of advanced cases as an aggregate. In other words, I personally believe that if residents are taught to perform 15 or 20 Nissen procedures, then they can go on and perform a laparoscopic gastric resection much more easily, and, similarly, if they can do a colectomy, then they can probably do a Nissen fundoplication without much difficulty because of the skills they have acquired. Did you have any sort of survey question where you asked about the minimum number of advanced cases to indicate advanced skills?

Dr. Park: The survey contained a question regarding the number of cases required to establish competency in basic procedures i.e., laparoscopic cholecstectomy and laparoscopic inguinal herniorrhaphy. Once these competencies were assumed the respondent then indicated how many cases of other procedures were needed. So each procedure was then taken as an isolated case once a basic level of training had been established.

Dr. Kenneth W. Warren

N. Tjarda van Heek, M.D., Kenneth W. Warren Surgical Research Fellow



Dr. Kenneth W. Warren meets the first Warren Surgical Fellow.

Most readers will remember Dr. Kenneth W. Warren as an internationally recognized surgeon asking critical questions at one of the gastrointestinal or pancreatic cancer meetings he loved to attend. His colleagues called him "the complete surgeon" since he combined a high degree of professional skill with personal concern for each patient. He always set goals for himself, the last of which was to launch the Kenneth W. Warren Surgical Research Fellowship. The week before he passed away on November 15, 2001, he insisted on meeting the first Warren Research Fellow. I am honored and fortunate to be that person, and I would like to share some of the last thoughts and remarks of this wonderful man with you.

At age 90 years, Dr. Warren was admitted to the New England Baptist Hospital where he had been Surgeon-in-Chief from 1969 to 1976, and a trustee since then. Although we had never met, he set me at ease at once with his warm-hearted personality. He was very alert in a sparkling way, and his stories about life were fascinating.

After I brought in his newspaper each morning and read the sport scores to him, Dr. Warren would sooner or later switch to his favorite topic: surgery. He told me amazing stories of patients and remembered names and details of their surgery, many of which were performed before I was born.

"Surgery is not difficult, nor scary, it is FUN." "Do you know how to prevent leakage from the pancreatic duct?" . . . he would order paper napkins and sutures, and demonstrate the perfect anastomosis. "The most common errors in the surgical treatment of acute pancreatitis are to operate too early in the course of the disease, and to do too much, or in the secondary or septic phase of the disease to operate too late and to do too little" (*Surgical Clinics of North America*, June 1964). Many of his surgical convictions he would express in a quotation.

–Every now and then he would take a nip of ginger ale–

Dr. Warren was fascinated by the anatomy of the peritoneum and was convinced that knowledge of the embryology of cleavage planes is the hallmark of a capable gastrointestinal surgeon. "Practice of gentleness to tissues is more important than the preaching." "But,"—a word often used by Dr. Warren, in a faint Southern accent— "the technique of an operation should be regarded as only a small part of the total care of a patient. My primary professional goal has always been: to get sick people well, and when that has proven impossible, to add to their comfort until the end." "Tjarda, travel the road together with your patients, don't be afraid of becoming friends with them, as long as you are in charge."

–nip of ginger ale–

"Could you fix my glasses, please, let's see how your surgical skills are."

From the Department of Surgery and Pathology (N.T.V.H.), Academic Medical Center Amsterdam, Amsterdam, The Netherlands; and the Department of Pathology (N.T.V.H.), The Johns Hopkins Medical Institutions, Baltimore, Maryland.
Reprint requests: N.T. van Heek, M.D., c/o Dr. John L. Cameron, 720 Rutland Avenue, 759 Ross Building, Baltimore, MD 21205.

Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(02)00029-X

Witnessing all the different people dropping by his room, I could tell that no one had been beneath his recognition. As a former trainee of Dr. Warren said, "He showed all people dignity and respect, not only as a physician, but that was the person he truly was."

Each nurse who walked in and out in the days I spent with him, he called his favorite. He made up for his occasional grumbling by showing them a big smile and making jokes. Humor was obviously very important to him, and he believed we should criticize our own behavior every day.

He encouraged me to pursue my pancreatic cancer research. "Early diagnosis and treatment is the key." He explained to me his belief in the obligation of physicians and surgeons to expand the frontiers of their field. "Young surgeons, like you, will open the doors of the future. Be skeptical, you should remember the immortal words of Alexander Pope, 'Be not the first by whom the new is tried, nor yet the last to lay the old aside'."

–nip of ginger ale–

"It disappoints me that you don't know anything about baseball. You Dutch only talk about soccer. You should be celebrating that the Yankees lost."

Sometimes he revealed a glimpse of his persistent character. He insisted on organizing a luncheon for me with the hospital staff in the boardroom that carries his name. He got himself into a wheelchair that day and gave a fabulous and funny speech, as he obviously had done many times during his life.

At the end of our four days together, he gave me his favorite book, "Embryology of the Peritonaeum" from 1892, his favorite pair of scissors, and a big hug. No words of goodbye were allowed, he continued reading his newspaper.

Dr. Kenneth Warren touched my professional and personal life, as he touched many others. I will cherish those days with him and continue my Fellowship in pancreatic cancer research at The Johns Hopkins Medical Institutions in his remembrance.

Investigators' Responsibilities and Rights

Based on recent reports,[1–9] there are increasing concerns about the control of the scientific data obtained from clinical trials sponsored by industry. Many of the problems encountered are the result of restrictions contained in the research contracts that participating investigators are asked to sign. A number of solutions have been suggested to ensure the integrity of clinical trials, including the establishment of appropriately constituted trial oversight committees, negotiating noninterference pledges from industry sponsors, and creating proactive support of investigators' rights by organized medicine.[10]

As surgical journal editors, we stand opposed to inappropriately restrictive contractual agreements governing company-sponsored clinical trials of devices or drugs, such as those containing clauses that deny the investigators proper control over the scientific aspects of the trial or restrict access to the data and its timely publication. We believe that responsibility for the scientific data from clinical trials and their analysis, interpretation, and publication should rest in the hands of the investigators. In multicenter trials, a duly appointed constituted publications committee can, and usually should, carry out these responsibilities.

Recently, the editors of 13 medical journals also published their opposition to inappropriately restrictive research contracts, insisting that investigators be given and assume adequate responsibility for the conduct of a clinical trial, have sufficient access to the data to perform the necessary analyses, and have control over the decision to publish. The editors further stated that they will "routinely require authors to sign a statement indicating that he or she accepts full responsibility for the conduct of the trial, had access to the data, and controlled the decision to publish," and "will not review or publish articles based on studies that are conducted under conditions that allow the sponsor to have sole control of the data or to withhold publication."[11]

Editors may choose not to publish an article if the sponsor had control over the trial design, data analysis, and/or publication. These requirements for publication ethics were adopted as policy on May 11, 2001, and will be included in the next publication of the *Uniform Requirements for Manuscripts Submitted to Biomedical Journals*—a document to which we are all signatories. The revised section on "Potential Conflicts of Interest Related to Project Support" is quoted here.

> Increasingly, biomedical studies receive funding from commercial firms, private foundations, and government. The conditions of this funding have the potential to bias and otherwise discredit the research.
>
> Scientists have an ethical obligation to submit creditable research results for publication. As the persons directly responsible for their work, researchers therefore should not enter into agreements that interfere with their access to the data or their ability to analyze the data independently, to prepare manuscripts, and to publish them. Authors should describe the role of the study sponsor(s), if any, in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the report for publication. If the supporting source had no such involvement, the authors should so state. Biases potentially introduced when sponsors are directly involved in research are analogous to methodological biases of other sorts; some journals therefore choose to include information about the sponsor's involvement in the methods section of the published paper.
>
> If a study is funded by an agency with a proprietary or financial interest in the outcome, editors may ask authors to sign a statement such as, "I had full access to all of the data in this study and I take complete responsibility for the integrity of the data and the accuracy of the data analysis." Editors should be encouraged to review copies of the protocol and/or contracts associated with project-specific studies before accepting such studies for publication. Editors may choose not to consider an article if a sponsor has asserted control over the authors' right to publish.

We, the undersigned surgical journal editors, support these revised guidelines and, as appropriate, will request authors of reports on clinical or basic research trials of devices and drugs to disclose details of the relative roles of the investigators and the sponsors in the conduct of the trial, the data collection and analysis, and preparation of the submitted manuscript. We may also ask the responsible author to sign a statement that he or she accepts full responsibility for the conduct of the trial, the validity of the data and its analysis, and the writing of the submitted manuscript.

American Journal of Surgery	Hiram C. Polk, Jr, M.D.
American Surgeon	Talmadge A. Bowden, Jr, M.D.
Annals of Surgery	Layton F. Rikkers, M.D.
Annals of Surgical Oncology	Charles M. Balch, M.D.
Archives of Surgery	Claude H. Organ, M.D.
British Journal of Surgery	John A. Murie, M.D.
Current Surgery	Walter J. Pories, M.D.

Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(02)00031-8

REFERENCES

1. Rutherford RB, Johnston KW. Potential problems with industry-supported research. J Vasc Surg 2000;31:1066–1076.
2. Weatherall D. Academia and industry: Increasingly uneasy bedfellows. Lancet 2000;355:1574.
3. Angell M. Is academic medicine for sale? N Engl J Med 2000;342:1516–1518.
4. Bodenheimer T. Uneasy alliance—clinical investigators and the pharmaceutical industry. N Engl J Med 2000;342:1539–1544.
5. Goldstein LB, Brott TG, Kothari RU, Smith WS. Clinical stroke trials: Guarding against bias. Stroke 1999;30:1165–1166.
6. Hailey D. Scientific harassment by pharmaceutical companies. Time to stop. CMAJ 2000;162:212–213.
7. Renni D. Fair conduct and fair reporting of clinical trials. JAMA 1999;282:1766–1768.
8. Kahn JO, Cheng DW, Mayer K, Murray H, Lagakos S. Evaluation of HIV-1 immunogen, an immunologic modifier, administered to patients infected with HIV having 300 to 549 $\times$ 10^6/L CD4 cell counts: A randomized controlled trial. JAMA 2000;284:2193–2202.
9. Blumenthal D, Campbell EG, Anderson MS, Causino N, Louis KS. Withholding research results in academic life science. Evidence from a national survey of faculty. JAMA 1997;277:1224–1228.
10. Rutherford RB, Johnston KW. Protecting the rights of investigators in industry supported clinical research. J Vasc Surg (in press).
11. Davidoff F, DeAngelis CD, Drazen JM, Nicholls MG, Hoey J, Hojgaard L. et al. Sponsorship, authorship, and accountability. N Engl J Med 2001;345:825.

Southwestern Center for Minimally Invasive Surgery (SCMIS): Laparoscopic Bariatric Surgery, June 21–22, 2002; September 27–28, 2002; The University of Texas Southwestern Medical Center at Dallas. Fees: physicians $300 (lecture only), $1050 (lecture and lab); UTSW and SCMIS Alumni $250 (lecture only), $950 (lecture and lab); nurse $175 (lecture only); $375 (lecture and lab). For further information contact: Jennifer Leedy, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9059. Phone: 214-648-3792; fax: 214-648-2317; e-mail: jennifer.leedy@utsouthwestern.edu

Southwestern Center for Minimally Invasive Surgery (SCMIS): Laparoscopic Bariatric Surgery Mini Fellowship Program, August 25–30, 2002; November 3–8, 2002; The University of Texas Southwestern Medical Center at Dallas. Fees: $10,000 (team of 2 physicians and 1 nurse); $5000 (physician); $1000 (nurse). For further information contact: Jennifer Leedy, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9059. Phone: 214-648-3792; fax: 214-648-2317; e-mail: jennifer.leedy@utsouthwestern.edu

AHPBA ANNOUNCEMENTS

November 2002: AASLD/AHPBA Surgical Forum, Boston, MA, November 1–5, 2002. This will be a liver-based program. Visit AHPBA.org for more information.

February 2003: 5th AASLD Biannual "Americas" Meeting, February 27–March 2, 2003. This will again be held at the Eden Roc Hotel in Miami, FL. The first day will be a postgraduate course chaired by C. Wright Pinson. Make this one with the family! Visit AHPBA.org for more information.

May 2004: 6th World Congress IHPBA Biannual Meeting, Washington, DC, May 27–June 2, 2004. The AHPBA hosts the IHPBA.

Published by Elsevier Science Inc.
1091-255X/02/$—see front matter
PII: S1091-255X(02)00032-X